SECTION 2.9.

OTHER DISEASES

CHAPTER 2.9.1.

BUNYAVIRAL DISEASES OF ANIMALS
(excluding Rift Valley fever)*

SUMMARY

A part of the large complex group of arboviruses is the Bunyaviridae family with over 300 members distributed over five genera. Two of the genera of veterinary importance in the family are Nairovirus, which contains the ruminant pathogen Nairobi sheep disease (NSD) virus, and the largest genus, Orthobunyavirus, which is subdivided into 48 serogroups. This genus contains only a few viruses that are significant pathogens of animals, among them Cache Valley virus (CVV) and Akabane virus. Although they have been placed in different antigenic groups, these two viruses have a tropism for fetal tissues and are responsible for embryonic and fetal losses and multiple congenital deformities in domestic ruminants. Members of the Nairovirus and Orthobunyavirus genera are single-stranded, enveloped spherical or pleomorphic RNA viruses, 80–110 nm in diameter. A third member of the Bunyaviridae family that is of veterinary importance is Rift Valley fever, a member of the Phlebovirus genus, and is discussed in Chapter 2.1.14.

Identification of the agent:

Cache Valley virus (CVV), a member of the Bunyamwera virus serogroup of the Orthobunyaviridae genus, can be isolated from the blood of febrile or viraemic adult animals. Attempts at isolation from the fetus at birth are generally unsuccessful due to virus clearance by the fetal immune response. Cell lines derived from monkey or baby hamster kidney are employed for isolation of the virus or alternatively, intracerebral inoculation of infant mice may be used. Virus or antigen is identified by fluorescence (FAT), immunohistochemistry (IHC) or neutralisation tests. Group- and virus-specific polymerase chain reaction (PCR) techniques have been developed for the Orthobunyaviruses.

Akabane virus can be isolated from the blood of viraemic animals and occasionally from fetal material. Cell lines of monkey, baby hamster and mosquito are used. The virus produces deformities in the developing chicken embryo. Yolk sac inoculation, as well as intracerebral inoculation of suckling mice, is used. Virus or antigen is identified by FAT, IHC or neutralisation tests. Nested and multiplex real-time reverse-transcriptase PCR techniques have been developed for Akabane and related viruses.

Nairobi sheep disease (NSD) virus is best isolated from plasma from febrile animals, mesenteric lymph nodes or spleen. Laboratory-reared sheep, 2–4-day-old unweaned mice inoculated intracerebrally, or cell cultures may be used for primary isolation. Sheep are the most sensitive animals for this, whereas a baby hamster kidney cell line and lamb or hamster kidney cell cultures are the most sensitive cells. Subinoculation of plasma from an experimentally infected sheep into cell cultures or mice is also recommended. Identification of the virus may be made by direct immunofluorescence of inoculated tissue cultures or of mouse brain smears. The agar gel immunodiffusion test can also be used to demonstrate the presence of NSD antigen in tissues but

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1 The diseases in this section that are marked with an asterisk are included in some individual species sections of the OIE List, but these Terrestrial Manual chapters cover several species and thus give a broader description.
will cross react with other viruses of the Nairovirus genus. Infected tissue cultures or mouse brain suspensions may be used as sources of complement-fixing or enzyme-linked immunosorbent assay (ELISA) antigens.

**Serological tests:** For CVV and Akabane virus, haemagglutination inhibition, complement fixation (CF) and serum neutralisation tests are used to detect antibodies. An ELISA, based on one used for Rift Valley fever, has also been described. A competitive ELISA specific for Akabane has been published. For NSD the most suitable test is the indirect fluorescent antibody test. CF and indirect haemagglutination tests have also been used to confirm NSD outbreaks in the field. Virus neutralisation tests give equivocal results, a feature that also occurs with other members of the Nairovirus group. ELISAs are now being evaluated for NSD. Infected spleen may be used as a source of antigen in immunodiffusion tests.

**Requirements for vaccines and diagnostic biologicals:** No vaccine is currently available for CVV. Vaccines for domestic use against Akabane virus have been produced in both Japan and Australia. For NSD, an experimental attenuated live virus vaccine has been investigated, and a killed tissue culture vaccine has been shown to be immunogenic.

### A. INTRODUCTION

- **Cache Valley virus**

Cache Valley virus (CVV) is a teratogenic Orthobunyavirus of North America affecting mainly sheep. A recent survey of cattle indicated that up to 28% were positive for specific antibodies to CVV (35). It is a member of the Bunyamwera serogroup of the Orthobunyavirus genus, family Bunyaviridae (19) and is also the most common of the Orthobunyaviruses of North America (4). CVV was first isolated from a mosquito pool in Utah, United States of America (USA) in 1956 (20), but was only linked to disease during an epizootic of neonatal loss and malformed lambs in a sheep flock in Texas in 1987 (9). The virus has also been isolated from a horse and a clinically healthy cow.

Serological surveys have shown a widespread prevalence of antibodies in domestic and wild ruminants and horses. Seroprevalence to CVV is high in deer, and the 1–3 day viraemia is sufficient to infect vectors allowing deer to act as amplifying hosts (2). Vectors include both Culicoides midges and mosquitoes of the Aedes, Anopheles, Coquillettidia and Culiseta groups.

CVV infection of adult animals is largely subclinical, and experimentally infected ewes show only a transient febrile response, but with a detectable viraemia. Human disease has been reported on two occasions (5, 36).

CVV was the first North American Orthobunyavirus to be linked to fetal arthrogryposis and hydranencephaly, however other related viruses have been shown experimentally to have the same potential. Fetal infection with CVV is age dependent in its outcome. Malformations take place between 27 and 45 days gestation, with infection at 28–36 days giving rise to central nervous system (CNS) and musculoskeletal defects, and infection at 37–42 days giving rise to musculoskeletal deformities only. Infection after 50 days gestation does not result in lesions and after 76 days the fetus is immunocompetent and antibodies are produced. Most CVV fetal deaths occur between 27 and 35 days gestation. The fetus is, however, susceptible at any age demonstrating the tropism of many Orthobunyaviruses for fetal tissues (6).

Gross pathology of the musculoskeletal system includes arthrogryposis of one or more limbs, torticollis, scoliosis of the vertebral column and muscular hypoplasia. CNS lesions include hydranencephaly, microencephaly, cerebral and cerebellar hypoplasia and micromelia (6, 18). Dead embryos and stillborn or mummified lambs with no obvious defects are also found. Anasarca is seen, as is oligohydramnion. This reduction in amniotic fluid is thought to contribute to restriction of fetal movement and thus to the skeletal deformities seen. Limb defects are also due to neurodegenerative changes seen histopathologically as areas of necrosis and loss of paraventricular neurupiloculi in the brain together with a reduction in the number of motor neurons. Skeletal muscle changes involve poorly developed myotubular myocytes (18).

- **Akabane virus**

Akabane virus is a teratogenic Orthobunyavirus widely distributed across the world but not in New World countries. It affects mainly cattle. It is a member of the Akabane serogroup, Orthobunyavirus genus, family Bunyaviridae (24). Other Orthobunyaviruses that are potential pathogens are Aino (Shuni virus serogroup), Peaton (Shamonda virus serogroup), Douglas (Sathupari virus serogroup) and Tinaroo (Akabane virus serogroup).
Akabane virus is, however, the best studied and most pathogenic of the Orthobunyaviruses and a major cause of arthrogryposis and hydranencephaly.

Akabane virus was first isolated in Japan in 1959, initially from a mosquito pool and then a pool of Culicoides midges. This was followed in 1972 by isolations from Culicoides in Australia and mosquito pool isolations in Africa. Akabane virus antibodies have been demonstrated in sera from cattle, sheep, goats, horses, buffalo and camels. Many indigenous game species in Africa south of the Sahara have Akabane virus neutralising antibodies. The range of Akabane virus includes the Middle East, Asia, Cyprus and Africa, but it is in Australia and Japan where regular epizootics of Akabane virus disease occur. Conditions favourable to such outbreaks are susceptible animals in early pregnancy and a sudden increase in vector populations, particularly when the virus has been absent from the area for a number of years.

Akabane virus infection in adult animals is usually subclinical, but encephalomyelitis has been recently associated with Akabane virus infection in adult cattle (28). Cattle seroconvert after a 3–4-day viraemia.

In endemic areas, antibody in the female animal prevents fetal infection, but Akabane virus is capable of establishing a long-term infection of the placenta in susceptible cattle and sheep. This takes place between 30 and 70 days gestation in the ewe and between 30 and 150 days gestation in the cow. Akabane virus has a predilection for brain, spinal cord and muscle cells where non-inflammatory necrosis interferes with morphogenesis.

Akabane virus infection has been studied experimentally in sheep and goats with the production of arthrogryposis/hydranencephaly, kyphosis, scoliosis, micro- and porencephaly, stillbirths and abortions (34). Natural infection of the ovine fetus has been described in Australia where perinatal lamb mortality and congenital microencephaly were most often seen.

Experimental Akabane virus studies have been carried out in pregnant cattle and it was shown that the type of abnormality is dependent on the gestational age of the fetus with hydranencephaly seen at 76–104 days and arthrogryposis at 103–174 days gestation (25). This time differential in appearance of abnormalities is clearly seen in bovine fetuses, whereas in sheep with a shorter gestation period, brain and skeletal lesions appear concurrently in the same fetus. The sequence of events during an epizootic of Akabane virus-induced fetal loss are the birth of uncoordinated calves, followed by those with arthrogryposis and dysplastic muscle changes, and lastly those with hydrocephalus and other severe CNS lesions. These events may be preceded by stillbirths and abortions (37). Akabane virus is responsible for severe neural and muscular abnormalities and lesions are characterised by a nonpurulent encephalomyelitis, focal cerebral degenerative encephalomyelopathy porencephaly, microencephaly, hydrocephalus, loss of ventral horn motor neurons and axons, depletion of myelin in spinal cord motor tracts, necrosis and polymyositis in the myotubules with parenchymal degeneration of skeletal muscles. Spinal cord abnormalities include scoliosis, and kyphosis and arthrogryposis may affect almost any skeletal joint.

- **Nairobi sheep disease**

Nairobi sheep disease (NSD) is a disease of sheep and goats caused by a Nairovirus of the family Bunyaviridae (11). It is characterised by a mortality rate, which may range between 40% and 90%, and should always be suspected when animals have recently been moved from an area free from the disease into one where it is endemic. Outbreaks also follow incursions of ticks into previously free areas, particularly following heavy rains (12). The clinical signs are similar in both sheep and goats, although there are differences in susceptibility among the various breeds and strains in their response to infection with NSD virus, some being more susceptible than others. Cattle and game are refractory to infection with NSD virus (43). The incubation period for the disease varies from 2 to 5 days, when a temperature reaction of 41–42°C develops. There is hyperventilation accompanied by severe depression, anorexia and a disinclination to move. Animals stand with lowered head, and show a conjunctivitis and sero-sanguinous nasal discharge. Some of the superficial lymph nodes, such as the prescapular and/or precrural, become palpable. Diarrhoea usually develops within 36–56 hours of the onset of the febrile reaction. This is at first profuse, watery and fetid, later haemorrhagic and mucoid, and accompanied by colicky pains and tenesmus. Abortion is a common sequel to the infection. Examination of the predilection sites for the attachment of ticks, such as the ears, head and body, is likely to reveal the presence of the ixodid tick *Rhipicephalus appendiculatus*.

Deaths can occur in peracute cases within 12 hours of the onset of the fever and at any time during the febrile reaction, while the animal is acutely ill. Further deaths then follow the fall in temperature for a further 3–7 days, associated with severe diarrhoea and dehydration.

The gross pathology of NSD can be misleading, for most deaths are likely to occur during the period of viremia, when the only signs are likely to be lymphadenitis with petechial and ecchymotic haemorrhages on the serous surfaces of the alimentary tract, spleen, heart and other organs. None of these signs allows a specific diagnosis of NSD to be made or even suspected, for they are shared with many other febrile diseases of sheep in NSD-endemic areas. Diseases with which NSD may be confused include Rift Valley fever, peste des petits ruminants,
rinderpest, salmonellosis and heartwater. Later in the course of the disease, a haemorrhagic gastroenteritis becomes more obvious, with haemorrhages on the mucosa of the abomasum, especially along the folds, in the region of the ileo-caecal valve, and most commonly in the colon and rectum. Zebra striping of the latter is often seen. The gall bladder is usually enlarged and haemorrhagic. Inflammatory lesions with haemorrhage may be seen in the female genital tract, if there has been abortion. However, in many animals dying from NSD, there may be none of these gastroenteric lesions, and a tentative diagnosis based on post-mortem signs can rarely be made. Common histopathological lesions are myocardial degeneration, nephritis and necrosis of the gall bladder.

The post-mortem signs following the early stage of NSD death are the nonspecific changes of congestion and petechial and ecchymotic haemorrhages on serous surfaces, on lymph nodes, the spleen and other organs such as the kidney, lungs and liver. Following death at later stages, haemorrhagic gastroenteritis becomes apparent, with ulceration of the abomasum, duodenum, caecum and colon. The virus is principally transmitted by the tick *Rhipicephalus appendiculatus*, and any infestation with such parasites should arouse suspicion of the presence of the disease. NSD virus may also be transmitted by other species of the genus *Rhipicephalus* and by the bont tick *Amblyomma variegatum*.

NSD is an apparently rare zoonotic agent in the field, causing mild influenza-like disease in humans. Laboratory infection has been associated with fever and joint pains (43).

### B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

   - **Cache Valley virus**

     At birth CVV cannot be isolated from the fetus but has been isolated from mosquito pools and the blood of viraemic adult animals. This has been done on tissue culture using hamster and monkey kidney cell lines including baby hamster kidney (BHK), African green monkey kidney (Vero) and LLC-MK2. Virus can be isolated from a febrile animal using a 10% buffy coat suspension in minimal essential medium (MEM) and co-cultivation with Vero cells in MEM supplemented with 2% fetal bovine serum.

     Virus isolation is also commonly done in newborn or weaned mice by intracerebral or intraperitoneal inoculation.

     Many *Orthobunyaviruses* have been sequenced as they are medically important pathogens associated with encephalitis in humans in both North and South America. Polymerase chain reaction (PCR) technology has been applied to mosquito-pool surveillance, instead of traditional isolation in infant mice, and sensitivity is reported to be one positive mosquito in a pool of 100, which is undetectable by traditional plaque titration in cell culture (21).

     Group-specific and virus-specific primers have been designed, and using the reverse-transcription PCR (RT-PCR) the Bunyamwera (BUN) and California (CAL) serogroup viruses can be distinguished. Using a nested RT-PCR technique the CAL and most of the BUN serogroup viruses can be distinguished from other *Orthobunyavirus* genus members (26, 31).

   - **Akabane virus**

     Diagnosis of infection is rarely made by virus isolation, but rather by histopathology and serology. Virus has however been isolated from viraemic sentinel animals using plasma or buffy coat suspensions from vector pools and occasionally from fetal material. RT-PCR has been described for the detection of Akabane virus and differentiation from Aino. Use of this assay could contribute to diagnosis, but the diversity of the *Orthobunyavirus* genus will require validation to confirm specificity of the test as there is evidence of reassortment.

     Suckling mice, 1–2 days old, may be used and inoculated intracerebrally with 0.01 ml of a clarified 10% suspension of the test material. A sensitive system for recovery of this serogroup of viruses is intravenous inoculation of embryonating chick eggs (ECE), followed by passage of harvested material in insect cells (C6/36) then mammalian cell lines (22, 42). Virus isolation in tissue culture is frequently done using Vero, BHK-21 and HmLu-1 cell lines. If C6/36 mosquito cells are used, cultures are left stationary for 7 days and material is repassaged on to a hamster or Vero cell line where cytopathic changes in the cultures become visible.

     Methods employed for specific identification of Akabane virus using monospecific antibodies have included virus neutralisation, and immunofluorescence (3, 22). Antigen detection in formalin-fixed material by peroxidase staining of bovine and ovine fetal material also in naturally infected newborn calves (33). Nucleic acid detection methods have also been developed for the differentiation of Aino and Akabane viruses using a nested RT-PCR technique.
followed by restriction enzyme digests to differentiate Akabane and Tinaroo viruses in the same serogroup and Aino and Peaton viruses in the Shuni and Shamonda serogroups (1). A multiplex real time RT-PCR has also been described using Taq Man probes which is said to reliably identify akabane and Aino viruses accurately (38).

- **Nairobi sheep disease virus**

NSD virus may be isolated from material collected from field cases by the use of laboratory animals or cell cultures (16). Safety precautions against aerosol infections should be taken when working with this agent. Uncoagulated blood, mesenteric lymph nodes and spleen tissue submitted with frozen gel packs are the optimal samples to be collected from febrile or dead animals. The plasma can be used directly as inoculum, and the lymph nodes or spleen should be homogenised to make an approximate 10% (w/v) suspension in a transport medium. This medium can be Hanks’ medium with 0.5% lactalbumin hydrosolate or 0.75% bovine serum albumin, and containing penicillin (500 International Units/ml), streptomycin sulphate (500 µg/ml), and mycostatin (50 units/ml) or fungizone (2.5 µg/ml).

A recommended initial procedure is to inoculate an NSD-susceptible sheep held in isolation with 1–2 ml of the tissue suspensions or plasma. Any pyrexia and clinical disease that develops permits a tentative diagnosis of NSD and, at the same time, furnishes excellent samples for virus isolation. This is especially valuable where the original field samples have been transported in hot climates where some loss of virus has inevitably occurred. Sheep are at least 100 times more sensitive than mice to NSD virus infection.

Infant mice, 2–4 days old, can be inoculated intracerebrally with 0.01 ml of a 1/10 dilution of plasma or of the tissue suspension. Two litters, each of 8–10 suckling mice, should be used for each sample and one blind passage is made routinely. The mice become debilitated and die within 5–9 days post-inoculation. Their brains should be harvested aseptically, pooled and diluted 1/100 for passage into additional mice.

Cell cultures may be used in conjunction with mouse inoculation for the primary isolation of NSD virus, as they have shown levels of sensitivity similar to that of the intracerebral inoculation of unweaned mice. The BHK-21-C13 cell line is especially valuable; the Vero cell line (37) and primary and secondary lamb or hamster kidney cells have also been used. Most strains of NSD virus produce a cytopathic effect (CPE) on first passage in BHK cells; others produce a more obvious CPE only after subinoculation. The appearance of a CPE is not such a regular finding with lamb testis and kidney cells, although it is usually seen on the second passage in lamb kidney cells. Tube cultures should be used both with and without flying cover-slips, or if plastic bottles are used for isolation, microwell slide cultures should also be prepared. Approximately 0.2 ml should be inoculated and a period of 1–2 hours allowed for adsorption. The CPE becomes evident in roller cultures as foci of granular rounded cells after 24–48 hours in BHK cells, and in a further 24–48 hours in other cell types. The CPE is not specific for NSD virus, which is identified in cover-slip cultures by immunofluorescence or by staining with haematoxylin and eosin. The latter method reveals pleomorphic eosinophilic intracytoplasmic inclusions peculiarly of a spindle form; other inclusions are bipolar, or surround the nucleus.

The virus can be specifically identified by immunofluorescence staining, which may be positive as early as 24–48 hours post-inoculation when no CPE has yet become evident. Conjugates for direct immunofluorescence may be prepared from hyperimmune mouse ascitic fluids, and from immune rabbit or sheep antisera by standard methods. Some cross-fluorescence may occur with other Nairoviruses at low dilutions of the conjugate, but these viruses are not normally associated with disease in sheep or goats.

The agar gel immunodiffusion test (AGID) can be a valuable primary diagnostic tool for the detection of NSD antigen in tissues. The test can be carried out in laboratories without tissue culture facilities and at field investigation laboratories. The spleen and mesenteric lymph nodes are the tissues of choice to be used in the test. Aliquots of 0.5–1 g should be homogenised with sterile sand in a pestle or a homogeniser to give 10–20% suspensions in phosphate buffered saline (PBS) or saline. The suspension should be centrifuged for 10–15 minutes at approximately 1000 g and the supernatant fluid is used in the test. This test can also be used for the identification of NSD virus antigen in mouse brain harvested from experimentally infected mice (see above). Rabbit hyperimmune serum against NSD can be prepared by repeated inoculation of NSD-infected mouse brain. A mouse brain suspension at 2–5% (w/v) is prepared as above and centrifuged at 3–5000 g for 15 minutes. Aliquots are then mixed with an equal volume of Egg Titremax adjuvant. Various inoculation regimes may be used but 1 ml volumes may be given subcutaneously and/or intramuscularly at 7-day intervals for 3–5 weeks, or at multiple inoculation sites in 0.1 ml volumes for a similar period. Serum should be collected 5–7 days after the last injection and stored in aliquots at −20°C.

Difco Noble or other suitable agar may be used in the test, using 0.85% sodium chloride at pH 7.2. Slides are prepared to give an agar layer approximately 2 mm in depth. Six wells should be placed hexagonally around a central well. The hyperimmune rabbit serum is placed in the central well and positive control antigen in wells 1 and 4. The tissue under test is placed in wells 2 and 5. Negative control tissue is placed in wells 3 and 6. Wells containing test tissue that give a precipitin line of continuity with the line formed between the positive antigen and the hyperimmune serum are considered to be positive.
Mouse brain suspensions or infective tissue culture fluids can be used as antigens for complement fixation (CF) tests for virus identification. Both have proved satisfactory after partial purification with fluorocarbon; the mouse brain can also be used in the form of a suspension in a borate buffer solution.

An enzyme-linked immunosorbent assay (ELISA) antigen for virus identification purposes can be prepared from an infected tissue culture in a bottle. The cells are removed using a pipette fitted with a rubber bulb when approximately 20% of the monolayer is showing CPE. They are sedimented and washed three times in borate saline buffer, pH 9. The cells are then lysed and solubilised with SDS (sodium dodecyl sulphate) and 1% Triton X100, diluted approximately 1/5 in borate saline buffer and sonicated to provide an antigen for the ELISA. A control negative antigen is prepared in the same manner from uninfected cells. These are adsorbed directly on to ELISA plates and the test is carried out with NSD immune and normal serum with both antigens.

2. Serological tests

These include haemagglutination inhibition (HI), CF and virus neutralisation (VN) tests and ELISA.

- **Cache Valley virus**
  a) **Virus neutralisation test**

  VN tests for CVV used to be done by a plaque reduction neutralisation method but are now usually performed using inhibition of CPE on Vero cells in microtitre plates (7).

  - **Test procedure**
    i) Inactivate test sera at 56°C for 30 minutes in a water bath.
    ii) Make serial twofold dilutions of the sera in MEM from 1/2 to 1/16 and incubate at 37°C for 60 minutes with an equal volume of 100 TCID\(_{50}\) (tissue culture infective dose) per ml of virus. Standard controls are prepared in a similar manner.
    iii) Discard the medium in a 96-well flat-bottomed cell-culture grade microtitre plate containing a preformed 24-hour Vero monolayer.
    iv) Add the serum/virus mixtures to the plate, 50 µl per well, using three wells per dilution.
    v) Back titrate the virus used in the test, making three tenfold dilutions using 50 µl per well and four wells per dilution.
    vi) Cover the plates and incubate for a further 60 minutes at 37°C.
    vii) Add 50 µl MEM maintenance medium to each well.
    viii) Incubate the plates at 37°C for 6 days in a humidified CO\(_2\) incubator.
    ix) Read the plates microscopically, evaluate the CPE and determine the 50% end points.
    x) The virus control should give a value of 100 TCID\(_{50}\) and there should be no neutralisation by the negative control serum at the lowest dilution tested. The positive control should give a titre within an expected range of its predetermined mean.

  b) **Enzyme-linked immunosorbent assay**

  An ELISA, modified and based on the one for Rift Valley fever described by Meegan et al. (30), has been used for CVV serological surveys. Modifications include a 1/400 dilution of mouse ascitic fluid to coat the plates, followed by a 1/25 dilution of a sucrose/acetone mouse brain antigen in a sandwich ELISA format. The diluent used is PBS with 0.5% Tween 20, 5% equine serum and 500 µg dextran sulphate per ml. A horseradish peroxidase conjugate detection system and an ABTS (2,2’-azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid) substrate are used (30).

c) **Other tests**

Not all members of the Bunyamwera group produce haemagglutinins, but a HI test has been described for CVV using a sucrose/acetone sucking mouse brain antigen and goose erythrocytes at pH 6.2. The test is said to lack sensitivity compared with a VN test, providing only 50% detection of antibodies. The CF test is little used because of extensive cross-reactivity within the Bunyamwera group.

- **Akabane virus**
  a) **Haemagglutination inhibition test**
The HI test is modified after Clarke & Casals 1958 (8) and better haemagglutination is achieved with an increased NaCl molarity. The test is also pH dependant. Sera are pretreated with kaolin or acetone and then heat inactivated at 56°C for 30 minutes. The test is performed using four units of sucrose/acetone-extracted mouse brain antigen, 0.3% red blood cells and borate buffer, pH 9 (23).

b) Virus neutralisation test

VN tests have been described using HmLu-1 cells in tube cultures or Vero and BHK cells in flat-bottomed 96-well microtitre plates (10, 42). Two techniques have been described with a serum/virus incubation period of 1 hour or incubation overnight before the addition of the cells.

- **Test procedure**
  i) Inactivate the test sera at 56°C for 30 minutes in a water bath.
  ii) Prepare serial twofold dilutions of the sera in Eagles medium from 1/2 to 1/16 in a 96-well flat-bottomed microtitre plate using duplicate wells and 25 µl per well. Standard controls are prepared in a similar manner.
  iii) Add 25 µl per well of virus in Eagles medium diluted to provide 200 TCID$_{50}$ per 50 µl.
  iv) Cover and incubate at room temperature for 1 hour.
  v) Include a back titration of virus in triplicate, making three tenfold dilutions using 25 µl per well.
  vi) Add 100 µl per well Vero cells in Eagles medium with 2% serum at 5 x 10$^5$ cells/ml.
  vii) Incubate the plates at 34–37°C for 5 days in a humidified CO$_2$ incubator.
  viii) Read the plates microscopically and calculate the titre as the reciprocal of the highest serum dilution completely inhibiting the CPE.
  ix) The virus and serum controls should give the expected results.

Where overnight incubation is used, duplicate twofold serial dilutions of inactivated serum are mixed with 100 TCID$_{50}$ of virus using 100-µl volumes in each case. Following incubation for 1 hour at 37°C and overnight at 4°C, 50 µl BHK cells is added to the test. The plate is examined at 3 and 5 days incubation at 37°C and checked for CPE.

c) Enzyme-linked immunosorbent assay

Akabane virus ELISAs, using both IgG and IgM, have been described. Coating antigen is 10$^6$ TCID$_{50}$ per ml of virus grown on HmLu-1 cells diluted in a 0.05 M carbonate/bicarbonate buffer, pH 9.6. The wash medium is PBS containing Tween 20 and alkaline phosphatase. Rabbit anti-bovine IgG and IgM conjugates are used (40).

A similar ELISA using horseradish peroxidase rabbit anti-bovine IgG conjugate has also been described.

A competitive ELISA with a 98% specificity has also been developed (39).

d) Complement fixation test

The CF test is not described here as it is a group specific test and is rarely used anymore.

- **Nairobi sheep disease**

a) Indirect fluorescent antibody test

The indirect fluorescent antibody test (FAT) is the most suitable test for use with members of the *Nairovirus* serogroup. There are, however, some cross-reactions, particularly with Dugbe virus and also with other members of the group, such as Congo–Crimean haemorrhagic fever virus (13). The NSD antibody titres by this method range from 1/640 to 1/10,240, and such titres are not obtained with immune sera to other members of the group (14).

The method has been used in epidemiological studies and to study the response to experimental vaccines. There do not appear to be any serological differences among the 40–50 isolates that have been examined.
An NSD I-34 strain\(^2\) was the virus usually used to prepare antigen, and this has been adapted to grow in BHK-21-C13 cells, after a series of passages.

The virus antigen in the cell substrate of choice may be grown in loose cover-slips, multiwell slides, Teflon-coated slides or microtitre plates for the test. A method using Teflon-coated slides is described.

- **Preparation of antigen slides**
  
  i) Wash and sterilise Teflon-coated slides. This is done briefly with a hot detergent that is used for tissue culture glassware in the laboratory, then three rinses in tap water for 30 minutes, each followed by similar rinses in distilled/deionised water. Slides are then placed in 70% alcohol for 10 minutes, removed with a sterile forceps and wrapped in greaseproof paper. They will then be found to be sterile, but further sterilisation in a microwave for two cycles of 5 minutes each is recommended.

  ii) Place these slides in sterile dishes using a sterile forceps; a square polystyrene type is better than the round variety.

  iii) Mix a suspension of BHK cells containing approximately 25,000 cells/ml in BHK growth medium (usually Eagles for BHK cells), and add 1000 TCID\(_{50}\) of NSD I-34 strain per ml. Mix by pipetting. Prepare some uninfected negative control slides.

  iv) Add the infected cells in 50 µl volumes (for the 12-well size) or as appropriate to the size of the Teflon wells. Replace the cover on the dishes and put into a humidified CO\(_2\) incubator or anaerobic container.

  v) Leave overnight for the monolayer to form. Then remove the plates from the incubator to a laminar flow cabinet, and flood with maintenance medium using a pipette to cover the slides to a depth of 2–3 mm. Return to the incubator.

  vi) Harvest the antigen slides just as foci of CPE become detectable. This will be in 36–56 hours (more accurate determination of the optimal harvesting time may be made by fixing and staining one slide after 24, 36 and 48 hours).

  vii) The slides are washed three times in PBS and dried. They are then fixed with dry heat (minimum 80°C) or with ice-cold acetone for 10 minutes. The slides are wrapped and may be stored at 4°C for 2–3 months, or at –20°C for 1–2 years. Slides stored at –20°C must be brought to 4°C overnight before use.

Similar procedures may be followed to prepare antigen on flying cover-slips or multiwell culture slides. When using Nunc tissue culture multiwell plates, however, fixation should be with 75% acetone.

- **Test procedure**

  i) Hydrate the slides by adding a drop of PBS to the wells with a Pasteur pipette. Number the slides according to the number of sera to be tested. Include in the series control positive and negative sera with infected and uninfected cell cultures.

  ii) Discard the PBS and add the serum dilutions 1/80–1/2560 in a predetermined manner to wells 1 to 6. It is preferable to duplicate each dilution on the same side.

  iii) Place the slides in dishes and hold at 37°C in a humid incubator for 40 minutes.

  iv) Wash the slides in racks in three changes of PBS, 5 minutes per wash.

  v) Add the fluorescein-conjugated anti-species conjugate (usually anti-sheep or anti-goat) at a predetermined working dilution; one drop can be added to each well with a Pasteur or other pipette.

  vi) Incubate as before for 30 minutes.

  vii) Wash three times in PBS and dry the slides.

  viii) Examine the slides by fluorescent microscopy. NSD virus antigen is found in the cell cytoplasm, and foci of bundles of fluorescing BHK cells will be seen. The antigen is seen mainly in fine fluorescent particles, but larger irregularly shaped antigen clumps occur, often surrounding the nucleus, or in spindle-like masses filling the cytoplasm to the pole of the cells. These particles will not be seen with negative sera or in the uninoculated control culture.

  ix) Sera that show this fluorescence at dilutions of 1/640 or 1/1280 are indicative of recent infection with NSD (14).

\(^2\) The I-34 strain was a virulent NSD isolate made in Kenya that was used extensively as a reference strain at the Kabete Laboratory – Kenya Agriculture Research Institute, P.O. Box 58137, Kabete, Nairobi, Kenya.
b) Other tests

CF tests are complicated by the marked anticomplementary activity of many sheep sera.

Immunodiffusion tests have been used successfully with crude antigens prepared from infected sheep tissue, tissue culture fluids or mouse brain material. Hyperimmune sera can be prepared in sheep, mice or rabbits for use in the test, using infected spleen taken from sheep dying from NSD as the source of antigen for immunisation.

An ELISA using a partially purified tissue culture antigen has been described for antibody testing and is suitable for use in serological surveys. The indirect FAT test should, however, be used to check doubtful results (32).

Monoclonal antibodies to the antigens of NSD virus strain I-34 have been developed and are being evaluated for their application as diagnostic reagents.

RNA probes have also been developed from the S (small) and M (medium) genome segments of Dugbe virus and have been used to demonstrate that the NSD serogroup of the genus Nairovirus is more closely related to the Crimean–Congo haemorrhagic fever serogroup than any of the remaining serogroups (29, 41). These probes also have the potential to be applied as diagnostic tools.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

- Cache Valley virus

Due to the sporadic nature of disease outbreaks, no vaccine has been developed.

- Akabane virus

Major epizootics of Akabane virus disease have only been reported from Japan and Australia, albeit at irregular intervals, but vaccination is seen to have merit in preventing fetal loss.

Vaccines for domestic use have been produced in Japan and Australia.

An inactivated vaccine is used in Japan for immunising cattle and goats. It is a formalin-inactivated intramuscular preparation with an aluminium phosphate gel adjuvant. Two 3-ml doses are given at a 4-week interval and yearly boosters are recommended. It is safe for use in pregnant animals. In field trials 88% of animals developed high VN antibodies after the first inoculation and there was a 100% response after the second dose (27). Similarly, in Australia an inactivated vaccine has been produced for intramuscular use giving two doses at a 4-week interval just before mating.

In Japan, a live Akabane virus vaccine is commercially available. A 1-ml dose is administered subcutaneously to cattle before the haematophagous arthropod vectors become active. Pregnant cattle and calves have been inoculated subcutaneously, intramuscularly and intracerebrally; no leukopaenia, viraemia or pyrexia were observed and a good VN antibody response was produced. A live Akabane virus vaccine, safe in cattle, was tested in pregnant ewes. During the trials, some ewes became viraemic and virus was found in the organs of several fetuses. Although no fetal deformities were produced, the vaccine is deemed unsuitable for use in sheep.

- Nairobi sheep disease

Epidemiological investigations have shown that in a state of enzootic stability, no problems are encountered with NSD. The disease arises from animal movements from free areas into endemic areas and can be avoided when such areas have been defined. Ecological changes that permit spread of the vector tick will result in extensions of these areas.

Experimental vaccines have been prepared for such situations. One vaccine consisted of virus attenuated by 35 passages in adult mice, but such vaccines can produce severe reactions in some breeds of sheep, and are not considered to be safe for general use. A similar vaccine was developed in Entebbe by further mouse brain passages, but this has not been further developed for use in the field in Uganda or elsewhere.

A tissue-culture-adapted strain of NSD virus has been grown to high titre in cultures grown in roller bottles. When precipitated with methanol, inactivated, and administered with an adjuvant, this was found to give good protection...
following two inoculations given at an interval of 14 days. Neither of these vaccines is routinely produced, for there has been little demand for their use from the field (15, 17).

REFERENCES


Chapter 2.9.1. — Bunyaviral diseases of animals (excluding Rift Valley fever)


