

SECTION 2.9.

OTHER DISEASES¹

CHAPTER 2.9.1.

BUNYAVIRAL DISEASES OF ANIMALS (excluding Rift Valley fever and Crimean–Congo haemorrhagic fever)*

SUMMARY

The Bunyaviridae family has over 300 members distributed over five genera. Viruses of all the genera except hantaviruses are transmitted to vertebrates by arthropods (arboviruses). The genera of veterinary importance are Nairovirus, Orthobunyavirus and Phlebovirus. Nairovirus contains the zoonotic Crimean–Congo haemorrhagic fever virus (for which see Chapter 2.1.5) and the ruminant pathogen Nairobi sheep disease (NSD) virus. The largest genus, Orthobunyavirus, is subdivided into 48 serogroups including only a few significant pathogens of animals, among them Cache Valley virus (CVV), Akabane virus (AKAV) and Schmallerberg virus (SBV). These viruses have a tropism for fetal tissues and are responsible for prenatal losses and multiple congenital deformities in domestic ruminants. SBV, a novel Orthobunyavirus, emerged in 2011 in Europe. The virus was found in malformed lambs, kids and calves in different European countries and spread to most parts of Europe. Other members of the Bunyaviridae family that are of veterinary importance are Rift Valley fever, a member of the Phlebovirus genus, described in Chapter 2.1.18 Rift Valley fever.

Members of the Nairovirus and Orthobunyavirus genera are enveloped spherical or pleomorphic RNA viruses, 80–110 nm in diameter, with three genome segments (S, M and L) of negative polarity.

Identification of the agent:

CVV, a member of the Bunyamwera virus serogroup of the Orthobunyavirus 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 African green monkey kidney (Vero) or baby hamster kidney (BHK) are employed for isolation of the virus or alternatively, intracerebral inoculation of infant mice may be used. Virus or antigen is identified by immunofluorescence (FA), immunohistochemistry (IHC) or neutralisation (VN) tests. Group- and virus-specific polymerase chain reaction (PCR) techniques have been developed for the Orthobunyaviruses.

AKAV can be isolated from the blood of viraemic animals and occasionally from fetal material. Vero, BHK and mosquito cell lines used. Virus or antigen is identified by FAT, IHC or VN tests. Nested and multiplex real-time reverse-transcriptase PCR (RT-PCR) techniques have been developed for Akabane and related viruses.

SBV can be isolated from the blood of viraemic adults, and occasionally from different tissues of infected fetuses, especially from brain material, using different cell lines: insect cells (KC), BHK or Vero. However, isolation can be difficult and adaptation to cell culture is necessary for in-vitro

¹ The diseases in this section of the *Terrestrial Manual* that are marked with an asterisk (in the Table of Contents and the chapter title) are included in some individual species sections of the OIE List, but these chapters cover several species and thus give a broader description.

growth of SBV. Real-time RT-PCRs have been established and commercial PCR kits are available allowing highly sensitive and specific virus detection in blood of acutely infected ruminants as well as in organs and blood of infected fetuses, such as brain, placenta, amniotic fluid, and meconium. Nevertheless, detection of SBV-genome is possible only in a proportion of the infected and malformed fetuses and not equally well in all tissue due to virus clearance during gestation.

Nairobi sheep disease (NSD) virus is best isolated from plasma from febrile animals, mesenteric lymph nodes or spleen. BHK cell line and lamb or hamster kidney cell cultures are the most sensitive cells. Identification of the virus may be made by FAT on inoculated tissue cultures. The agar gel immunodiffusion test can also be used to demonstrate the presence of NSD antigen in tissues but will cross react with other viruses of the Nairovirus genus. Infected tissue cultures may be used as sources of complement-fixing or enzyme-linked immunosorbent assay (ELISA) antigens.

Serological tests: For CVV and AKAV, ELISA and VN tests are used to detect antibodies. A competitive ELISA specific for Akabane has been published and commercial kits are available. For SBV, ELISA (commercial indirect and blocking ELISAs are available), indirect immunofluorescent antibody (IFA) and VN tests are used to detect antibodies against SBV in serum samples. For NSD the most suitable test is the IFA. Complement fixation (CF) and indirect haemagglutination tests have also been used to confirm NSD outbreaks in the field. VN 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 against AKAV have been produced. For NSD, an experimental attenuated live virus vaccine has been investigated, and a killed tissue culture vaccine has been shown to be immunogenic. In 2013, inactivated vaccines against SBV received provisional marketing authorisation in some European countries such as the United Kingdom and France.

A. INTRODUCTION

Bunyaviruses vary in their capability to infect humans, as indicated in the following description of each virus. Specific risk assessments as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities* should be carried out to determine both the biosafety and biocontainment measures required for handling infective materials in the laboratory.

1. Cache Valley virus

Cache Valley virus (CVV) is a teratogenic *Orthobunyavirus* of North America affecting mainly sheep. Experimental infection of ovine fetuses has confirmed the role of CVV in causing malformation (Rodrigues Hoffman *et al.*, 2012). A recent survey of cattle indicated that up to 28% were positive for specific antibodies to CVV (Sahu *et al.*, 2002). It is a member of the Bunyamwera serogroup of the *Orthobunyavirus* genus, family *Bunyaviridae* (Fauquet *et al.*, 2005) and is also the most common of the *Orthobunyaviruses* of North America (Calisher *et al.*, 1986). CVV was first isolated from a mosquito pool in Utah, United States of America (USA) in 1956 (Holden & Hess, 1959), but was only linked to disease during an epizootic of neonatal loss and malformed lambs in a sheep flock in Texas in 1987 (Crandell *et al.*, 1989). 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 (Blackmore & Grimstad, 1998). 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 (Campbell *et al.*, 2006; Sexton *et al.*, 1997).

CVV was the first North American *Orthobunyavirus* to be linked to fetal arthrogryposis and hydrancephaly, however, other related viruses have been shown experimentally to have the same potential. The clinical outcome of fetal infection with CVV is age dependent. 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 (Chung *et al.*, 1990).

Gross pathology of the musculoskeletal system includes arthrogyposis of one or more limbs, torticollis, scoliosis of the vertebral column and muscular hypoplasia. CNS lesions include hydrancephaly, hydrocephalus, porencephaly, microencephaly, cerebral and cerebellar hypoplasia and micromelia (Chung *et al.*, 1990; Edwards *et al.*, 1997). 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 neurophilis in the brain together with a reduction in the number of motor neurons. Skeletal muscle changes involve poorly developed myotubular myocytes (Edwards *et al.*, 1997).

2. Akabane virus

Akabane virus (AKAV) is a teratogenic *Orthobunyavirus* widely distributed across the world but not in the Americas. It affects mainly cattle. It is a member of the Simbu serogroup², *Orthobunyavirus* genus, family *Bunyaviridae* (Karabatsos, 1985). Other potential pathogens in the Simbu serogroup include Aino, Peaton, Schmallenberg, Shamonda and Tinaroo viruses. AKAV is a major cause of arthrogyposis and hydrancephaly. Experimental infections of neonatal calves and pregnant ewes demonstrated that Aino and Peaton viruses may cause malformations in ruminants (Parsonson *et al.*, 1982; Tsuda *et al.*, 2004), however Peaton virus has never been associated with disease under field conditions. Aino virus has caused outbreaks of congenital abnormalities in ruminants in Japan and once in Australia.

AKAV was first isolated 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. AKAV 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 AKAV neutralising antibodies. The range of AKAV includes the Middle East, Asia, Cyprus, Africa and Australia. Epizootics of Akabane disease occur sporadically in countries such as Australia and Israel where vaccination is not routinely practised. Outbreaks usually occur when conditions are favourable for vectors and they move beyond the endemic range into populations of susceptible animals in early to mid-pregnancy or when the virus has been absent from an endemic area for one or more years, usually as a result of drought.

AKAV infection in adult animals is usually subclinical, but encephalomyelitis has been associated with AKAV infection in adult cattle in Japan (Lee *et al.*, 2002; Miyazato *et al.*, 1989). Cattle seroconvert after a 3–4-day viraemia.

In endemic areas, females are infected prior to reaching breeding age and antibody in the female prevents fetal infection. Generally disease can be observed in the fetus of naïve dams following infection between 30 and 70 days gestation in the ewe or between 70 and 150 days gestation in the cow. At later stages of gestation, congenital defects are mild and uncommon although infection of the bovine fetus with some strains of AKAV close to term may result in the birth of calves with an encephalitis. AKAV has a predilection for brain, spinal cord and skeletal muscle cells where non-inflammatory necrosis interferes with morphogenesis.

AKAV infection has been studied experimentally in sheep and goats with the production of arthrogyposis/hydroencephaly, kyphosis, scoliosis, micro- and porencephaly, stillbirths and abortions (Parsonson *et al.*, 1975). Natural infection of the ovine and caprine fetus has been described where perinatal lamb mortality and congenital microencephaly were most often seen.

Experimental AKAV 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 hydrancephaly seen at 76–104 days and arthrogyposis at 103–174 days gestation (Kirkland *et al.*, 1988). The 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 fetus. The sequence of events during an epizootic of AKAV-induced fetal loss are the birth of uncoordinated calves, followed by those with arthrogyposis and dysplastic muscle changes, and lastly those with hydrancephaly and other severe CNS lesions. These events may be preceded by stillbirths and abortions (Shepherd *et al.*, 1978). AKAV is responsible for severe neural and muscular abnormalities and lesions are characterised by a nonpurulent encephalomyelitis, focal cerebral degenerative encephalomyelopathy porencephaly, microencephaly, hydrancephaly, loss of ventral horn motor neurons and axons, depletion of myelin in spinal cord motor tracts, necrosis and polymyositis in the myotubules with

2 The current classification by the International Committee on Taxonomy of Viruses does not recognise the term “serogroup” for bunyaviruses. It is used in this chapter as a term of convenience.

parenchymal degeneration of skeletal muscles. Spinal cord abnormalities include scoliosis, and kyphosis and arthrogryposis may affect almost any skeletal joint.

3. Schmallenberg virus

SBV was first detected in November 2011 in Germany from samples collected in October 2011 from dairy cattle with fever and reduced milk yield. Similar clinical signs (including diarrhoea) were detected in dairy cows in the Netherlands, where the presence of SBV was also confirmed in December 2011. From early December 2011, congenital malformations were reported in newborn lambs in the Netherlands, and SBV was detected in and isolated from the brain tissue. Since then, SBV has been detected in many European countries as well as in Kazakhstan. Suspicion of past infection has also been reported in Turkey and South Africa.

SBV is an enveloped, negative-sense, segmented, single-stranded RNA virus. It belongs to the *Bunyaviridae* family, within the *Orthobunyavirus* genus and is a member of the Simbu serogroup viruses, which includes Sathuperi, Akabane, and Aino viruses (Hoffman *et al.*, 2011). A recent phylogenetic analysis of several viruses of the Simbu serogroup suggested that SBV is not a reassortant and is most related to the Sathuperi virus species. A closely related virus within this species is the Australian Douglas virus (Goller *et al.*, 2012). Of note, viruses from the Simbu serogroup had never been isolated in Europe before 2011.

Like the genetically related viruses of the Simbu serogroup, SBV affects ruminants. Infection of cattle, sheep, goats, roe deer and bison has been confirmed both by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) and virus isolation (Bilk *et al.*, 2012; Hoffmann *et al.*, 2011). Antibodies were found in red deer, alpaca and mouflons.

Experimental infection in cattle and sheep showed no clinical signs or mild signs at 3–5 days post-inoculation with an incubation period of 2–4 days and viraemia lasting for 2–5 days (Hoffmann *et al.*, 2011, Wernike *et al.*, 2013)

Transmission is by insect vectors and then vertically *in utero*. SBV genome was detected in several *Culicoides* species. Further information is required to determine which are the competent insect species and whether mosquitoes play a role. Vertical transmission across placenta is proven but direct contamination from animal to animal is very unlikely. Experimental infection was not successful via the oral route and also contact animals were not infected (Wernike *et al.*, 2013). Furthermore, re-infection of previously infected calves was not possible (Wernike *et al.*, 2013).

Manifestation of clinical signs varies by species: bovine adults have shown a mild form of acute disease during the vector season, congenital malformations have affected more species of ruminants (to date: cattle, sheep, goat and bison). Some dairy sheep and cow farms have also reported diarrhoea (Beer *et al.*, 2012; Hoffmann *et al.*, 2012; van der Brom *et al.*, 2012; Wernike *et al.*, 2013).

The signs can be summarised as arthrogryposis and hydranencephaly syndrome (AG/HE): in malformed animals and stillbirths (calves, lambs, kids) the pathological signs were arthrogryposis-hydranencephaly, brachygnathia inferior, ankylosis, torticollis, scoliosis. The exact rate of malformation is not known and varies depending on the stage of gestation at the time of infection. Epidemiological, immunological and virological investigations are ongoing in several European countries.

Serological studies in humans did not show any evidence that it is a zoonotic disease (European Centre for Disease Prevention and Control, 2012; Reusken *et al.*, 2012).

4. Nairobi sheep disease virus

Nairobi sheep disease (NSD) is a disease of sheep and goats caused by a *Nairovirus* of the family *Bunyaviridae* (Davies, 1988). 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 (Davies, 1997). 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 (Zeller & Bouloy, 2000). 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 viraemia, 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 *Ambylomma 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 (Zeller & Bouloy, 2000).

B. DIAGNOSTIC TECHNIQUES

B1. Identification of the agent

1. Cache Valley virus

At birth CVV cannot be isolated from the neonate but has been isolated from mosquito pools and the blood of viraemic adult animals. This has been done on tissue culture using cell lines such as baby hamster kidney (BHK), African green monkey kidney (Vero) and adult Rhesus monkey kidney (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 (Huang *et al.*, 2001).

Group-specific and virus-specific primers have been designed, and using the 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 (Kuno *et al.*, 1996; Moreli *et al.*, 2001). A recent duplex real time RT-PCR for the CAL serogroup and CVV has been reported (Wang *et al.*, 2009) although not yet validated for veterinary applications.

2. Akabane virus

Diagnosis of infection is rarely made by virus isolation, but usually by serology and sometimes histopathology. 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 AKAV. 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.

Virus isolation in tissue culture is frequently done using Vero, BHK-21 and HmLu-1 cell lines. If C6/36 mosquito or KC *Culicoides* cells are used, cultures are left stationary for 7 days and material is re-passaged on to a BHK or Vero cell line where cytopathic changes in the cultures become visible.

Methods employed for specific identification of AKAV using monospecific antibodies have included virus neutralisation (VN), and immunofluorescence (FA) (Blacksell *et al.*, 1997; Gard *et al.*, 1988). Antigen detection in formalin-fixed material by peroxidase staining of bovine and ovine fetal material also in naturally infected newborn calves (Noda *et al.*, 2001). 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, Aino, Peaton and Tinaroo viruses in the Simbu serogroup (Akashi *et al.*, 1999). A multiplex real-time RT-PCR has also been described using Taq Man probes that is said to identify Akabane and Aino viruses reliably and accurately (Stram *et al.*, 2004). An alternative to RT-PCR for detection of AKAV uses reverse transcription loop-mediated isothermal amplification and can detect as little as five TCID₅₀ (tissue culture infective dose) of virus per ml (Qiao *et al.*, 2013).

3. Schmallenberg virus

SBV can be detected by real-time RT-PCR (Bilk *et al.*, 2012). Several commercial PCR kits are also available. Infectious virus can be isolated in cell culture. Insect cells (KC), hamster cells (BHK), monkey kidney cells (Vero) have been used. Samples for virus detection or isolation should be transported cooled or frozen. Serum or EDTA (ethylenediaminetetraacetic acid) blood are the usual source for the detection of acute infection in live animals during the short period of viraemia (2–6 days). For stillborns and malformed calves, lambs and kids, viral RNA can be detected in tissue samples of brain (cerebrum and brainstem) and amniotic fluid, and in amniotic fluid and placenta or meconium from live newborns. However, virus isolation is difficult and was only partially successful in trials.

For stillborns and malformed calves, lambs and kids diagnosis can be also performed by histopathology on fixed central nervous system, including spinal cord. The lesions are characteristic of hydranencephaly, hypoplasia of the central nervous system, porencephaly and subcutaneous oedema (calves). However, sensitivity is lower than with RT-PCR methods.

As the signs are not specific, differential diagnosis should be performed. For the acute infection of the adults, all sources of high fever, diarrhoea and milk reduction should be taken into account. For the malformation of calves, lambs and kids, other orthobunyaviruses, bluetongue virus, pestiviruses, genetic factors and toxic substances should be considered.

4. Nairobi sheep disease virus

NSD virus may be isolated from material collected from field cases by the use of laboratory animals or cell cultures (Davies *et al.*, 1977a). 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 hydrolysate 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).

For virus isolation in cell culture, the BHK-21-C13 cell line is especially valuable; the Vero cell line (Shepherd *et al.*, 1978) 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. CPE is not reliably produced on lamb testis or kidney cells, but may occur after multiple passage. 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 FA 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 FA 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 for use in the AGID 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 3000–5000 **g** for 15 minutes. Aliquots are then mixed with an equal volume of 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.

Infective tissue culture fluids can be used as antigens for complement fixation (CF) tests for virus identification.

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.

An NSD-specific real-time RT-PCR has been described and is reportedly more sensitive than virus isolation (Bin Tarif *et al.*, 2012). However it has not yet been fully validated for diagnostic use.

B2. Serological tests

These include haemagglutination inhibition (HI), CF, VN tests and ELISA, however HI and CF tests are rarely used.

1. Cache Valley virus

1.1. 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 (Chung *et al.*, 1990).

1.1.1. 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 TCID₅₀ 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 CO₂ 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 TCID₅₀ 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.

1.2. Enzyme-linked immunosorbent assay

An ELISA, modified and based on the one for Rift Valley fever described by Meegan *et al.* (1987), 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 (Meegan *et al.*, 1987).

1.3. Other tests

Not all members of the Bunyamwera group produce haemagglutinins, but a HI test has been described for CVV using a sucrose/acetone suckling 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.

2. Akabane virus

2.1. Haemagglutination inhibition test

The HI test is modified after Clarke & Casals (1958) and better haemagglutination is achieved with an increased NaCl molarity. The test is also pH dependent. 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 (Goto *et al.*, 1978).

2.2. Virus neutralisation test

VN tests have been described using HmLu-1, Vero or BHK cells in flat-bottomed 96-well microtitre plates (Cybinski *et al.*, 1976; Da Costa Mendes, 1984). Two techniques have been described with a serum/virus incubation period of 1 hour or incubation overnight before the addition of the cells.

2.2.1. 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 TCID₅₀ 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 cell suspension in Eagles medium with 2% serum at 5×10^5 cells/ml.
- vii) Incubate the plates at 34–37°C for 5 days in a humidified CO₂ 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 TCID₅₀ of virus using 100-µl volumes in each case. Following incubation for 1 hour at 37°C and overnight at 4°C, 50 µl cell suspension is added to the test. The plate is examined at 3 and 5 days incubation at 37°C and checked for CPE.

2.3. Enzyme-linked immunosorbent assay

AKAV ELISAs, using both IgG and IgM, have been described. Coating antigen is 10⁶ TCID₅₀ 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 (Ungar-Waron *et al.*, 1989).

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 (Tsuda *et al.*, 2004) and is available as a commercial kit.

3. Schmallenberg virus

Serological tests are performed on serum or plasma samples. The most currently used are i) ELISAs with in-house reagents or with the several kits (indirect or competition) that are now commercially available from several companies; ii) indirect Immunofluorescence test; and iii) the VN test.

In a first international interlaboratory comparison, the VN test was more sensitive than most of the ELISA systems used (van der Poel *et al.*, 2014).

3.1. Enzyme-linked immunosorbent assay

Several types of ELISAs have been developed (Breard *et al.*, 2013; van der Heijden *et al.*, 2013), and are also commercially available from different companies.

The systems can be divided into indirect ELISAs on the basis of recombinant N-protein or full-virus preparations, and competition ELISAs using nucleoprotein-specific monoclonal antibodies.

3.2. Virus neutralisation test

VN tests have been described using Vero or BHK cells in flat-bottomed 96-well microtitre plates (Loeffen *et al.*, 2012; Mansfield *et al.*, 2013).

The following protocol is based on Wernike *et al.* (2013). The neutralisation test is routinely performed with serum in 96-well microtitre plates using cell culture medium with antibiotics. Exceptionally, if no serum is available, the test can be performed with plasma, but in this case, dilutions <1/20 cannot be effectively evaluated.

3.2.1 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 medium from 1/5 to 1/640 in a 96-well flat-bottomed microtitre plate using duplicate or quadruplicate wells and 50 µl per well.

Load the wells of the first row with 80 µl, the wells of the other rows with 50 µl culture medium. Add 20 µl serum sample to the first row. Take 50 µl of the first dilution step (1/5 dilution), add to the next row, mix, and continue the dilution series. Discard the last 50 µl. Each well now contains 50 µl of a serum-medium dilution.

Standard controls (positive and negative reference sera) are prepared in a similar manner.

- iii) Add 50 µl per well of a virus preparation with 100 TCID₅₀/50µl (2000 TCID₅₀/ml). The required amount of test virus (approx. 5 ml per microtitre plate) should be prepared in one batch.

In addition to positive and negative serum controls, a cell control (100 µl culture medium without serum and virus), as well as a virus-free serum control (dilution 1/5) should be prepared.

A back titration of the test virus should be performed in each test. The virus dilution used in the test is diluted at log-2-steps in duplicate or quadruplicate, beginning with 1/10 to 1/1280. Wells are pre-filled with 180 µl culture medium, and 20 µ test virus suspension at a dilution of 1/10 are added and finally diluted.

- iv) Cover and incubate the microtitre plate for 2 hours at 37°C in a humid environment in a CO₂-cabinet. During this time the neutralisation process takes place.

- v) After the incubation period, add 100 µl of the respective cell suspension to each well. Adjust the cell density, so that after 24 hours a confluent cell layer develops. The microtitre plate remains in a humid environment in the CO₂-cabinet for incubation.
- vi) Incubate the plates at 34–37°C for 3–4 days in a humidified CO₂ incubator.
- vii) Evaluation is done by assessment of the cytopathic effect. The final read-out is done at day 3 or 4 after preparation of the test.

The test is valid, if the back titration ranges between 30 and 300 TCID₅₀, and the positive control serum shows the indicated titre (± one log₂-step).

The antibody titre is calculated as ND₅₀ according to Behrens and Kärber.

Neutralisation titre = $V-d \times (S-0.5)$

V: lg of the first 100% positive serum dilution

d: lg of the dilution factor (as a rule 0.3)

S: sum of the positive reagents from 0 to 100%/reagent number per dilution

4. Nairobi sheep disease virus

4.1. 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 Crimean–Congo haemorrhagic fever virus and Kupe virus (Davies *et al.*, 1978). 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 (Davies *et al.*, 1976).

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 strain³ 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.

4.1.1. 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 Eagles³ for BHK cells), and add 1000 TCID₅₀ 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 CO₂ 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.

³ 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 14733 – 00800 Nairobi, Kenya.

- 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.

4.1.2. 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 (Davies *et al.*, 1976).

4.2. Other tests

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

AGID 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 IFA test should, however, be used to check doubtful results (Munz *et al.*, 1984).

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 (Marriott *et al.*, 1990; Ward *et al.*, 1992). These probes also have the potential to be applied as diagnostic tools.

C. REQUIREMENTS FOR VACCINES

1. Cache Valley virus

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

2. Akabane virus

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

An inactivated vaccine is used for immunising cattle and goats. Either formalin or beta propiolactone-inactivated intramuscular preparations with an aluminium phosphate gel adjuvant have been used. Two 3-ml doses are given at a 4-week interval just before mating, 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 (Kurogi *et al.*, 1978). A high level of efficacy has been shown following natural challenge under field conditions.

In Japan, a live AKAV 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 AKAV 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.

3. Aino virus

Inactivated Aino virus vaccines have been developed and are commercially available in Japan. An inactivated trivalent vaccine (Aino, Akabane and Chuzan viruses) has been tested successfully in cattle (Kin *et al.*, 2011).

4. Schmallenberg virus

Inactivated SBV vaccines are available and are licensed in some European countries.

5. Nairobi sheep disease virus

Epidemiological investigations have shown that outbreaks of NSD do not occur in a state of enzootic stability. 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 (Davies *et al.*, 1974; 1977b).

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For further information, reference material and advice on Schmallerberg virus,
refer to the Friedrich-Loeffler-Institut, Insel Riems, Germany,