SECTION 3.10.

OTHER DISEASES

CHAPTER 3.10.1.

BUNYAVIRAL DISEASES OF ANIMALS (EXCLUDING RIFT VALLEY FEVER AND CRIMEAN–CONGO HAEMORRHAGIC FEVER)

SUMMARY

The order Bunyavirales *has hundreds of members distributed over 12 families with a large number of genera. Most viruses of the different families are transmitted to vertebrates by arthropods* (*arboviruses*). *Members of the family* Hantaviridae *are not arboviruses.*

The families of veterinary importance are Nairoviridae, Peribunyaviridae *and* Phenuiviridae. *The genus* Orthonairovirus *contains the zoonotic Crimean–Congo haemorrhagic fever virus* (*for which see Chapter 3.1.5*) *and the ruminant pathogen Nairobi sheep disease* (*NSD*) *virus* (*NSDV*). *The largest genus,* Orthobunyavirus, *is subdivided into 103 virus species and 48 serogroups including only a few significant pathogens of animals, among them Cache Valley virus* (*CVV*), *Akabane virus* (*AKAV*), *Schmallenberg virus* (*SBV*) and *Shuni virus* (SHUV). *These viruses have a tropism for fetal tissues and are responsible for prenatal losses and multiple congenital deformities in domestic ruminants. SBV is a novel* Orthobunyavirus *that 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* order Bunyavirales *that are of veterinary importance are Rift Valley fever virus* (*RVFV*), *a member of the* Phenuiviridae *family* (*genus* Phlebovirus), *described in Chapter 3.1.20* Rift Valley fever.

Members of the Orthonairovirus *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.*

Detection and 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 to isolate 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. Virus or antigen is identified by immunofluorescence* (*FA*), *immunohistochemistry* (*IHC*) *or neutralisation* (*VN*) *tests. Group- and virus-specific reverse transcription polymerase chain reaction* (*RT-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 can be used. Virus or antigen is identified by FAT, IHC or VN tests. Different types of real-time RT-PCR techniques have been developed and validated for AKAV and related viruses.

SBV can be isolated from the blood of viraemic adults, and occasionally from different tissues of infected fetuses, especially from brain/CNS materials, using different cell lines: insect cells (*KC; C6/36*) or mammalian *BHK or Vero cells. However, isolation can be difficult and adaptation to cell culture is necessary for sufficient* in-vitro *growth of SBV. Several 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 tissues due to virus clearance during gestation.

NSDV is best isolated from the plasma of febrile animals, mesenteric lymph nodes or spleen. BHK cells and lamb cell cultures are the most sensitive cells for isolation. Identification of the virus may be made by FAT on the inoculated tissue cultures. Infected tissue cultures may be used as sources of complement-fixing or enzyme-linked immunosorbent assay (ELISA) antigens. However, as for CVV, AKAV and SBV, real-time RT-PCR is the most sensitive and reliable detection technique and several protocols have been developed and validated.

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 immunofluorescence antibody (IFA) and VN tests are used to detect antibodies against SBV in serum samples. For NSDV a suitable test is the IFA, VN tests give equivocal results, a feature that also occurs with other members of the* Nairovirus *group. ELISAs are now also being developed and evaluated for NSD.*

Requirements for vaccines and diagnostic biologicals: *No vaccine is currently available for CVV. Vaccines against AKAV have been produced and has been used e.g. in Japan. For NSDV, an experimental attenuated live virus vaccine has been investigated, and a killed tissue culture vaccine has been shown to be immunogenic. Against SBV, several types of vaccines have been developed (modified live vaccines, vector vaccines, subunit vaccines and inactivated vaccines), and inactivated vaccines are authorised in Europe.*

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* (family *Peribunyaviridae*) of the Americas affecting mainly pregnant sheep and goats. Human illness has rarely been reported. Experimental infection of ovine fetuses has confirmed the role of CVV in causing malformation (Rodrigues Hoffman *et al.,* 2012). It is 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, 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 CVV antibodies in domestic and wild ruminants and horses, for example Uehlinger *et al.* (2018) found positivity rates in Canada of 20% in cattle, 33% goats, 69% horses and 51% mule deer. 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 hydranencephaly, 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 of 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 of 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 arthrogryposis of one or more limbs, torticollis, scoliosis of the vertebral column and muscular hypoplasia. CNS lesions include hydranencephaly, hydrocephalus, porencephaly, microencephaly, cerebral and cerebellar hypoplasia and micromelia (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 neutrophils 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, sheep and goats. Antibodies to AKAV have also been found in horses, donkeys, buffalo, deer, camels and pigs, but there are no reports of AKAV-associated illness in these species. It is a member of the Simbu serogroup[1], *Orthobunyavirus* genus, family *Peribunyaviridae*. Other potential pathogens in the Simbu serogroup include Aino, Peaton, Schmallenberg, Shuni, Shamonda and Tinaroo viruses. AKAV is a major cause of arthrogryposis and hydranencephaly. Experimental infections of neonatal calves and pregnant ewes demonstrated that Aino and Peaton viruses may also cause malformations in ruminants (Parsonson *et al.,* 1982; Tsuda *et al.,* 2004a). Aino virus has caused outbreaks of congenital abnormalities in ruminants in Japan and 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 pools 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 (Kirkland, 2015). Ruminants seroconvert after a 3- to 7-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 arthrogryposis/ hydranencephaly, 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 hydranencephaly seen at approximately 80–105 days and arthrogryposis at about 105–170 days gestation (Kirkland 2015). 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 arthrogryposis and dysplastic muscle changes, and lastly those with hydranencephaly 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,

1 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.

porencephaly, microencephaly, hydranencephaly, 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.

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 and Western Asian countries. Suspicion of past infection has also been reported in Africa.

SBV belongs to the *Peribunyaviridae* family, within the *Orthobunyavirus* genus and is a member of the Simbu serogroup (Hoffman *et al.,* 2011). Of note, viruses from the Simbu serogroup had never been detected in Europe before 2011.

Like the genetically related viruses of the Simbu serogroup, SBV affects ruminants. Infection of cattle, sheep, goats, roe deer, mouflon and bison has been confirmed by real-time reverse-transcription polymerase chain reaction (RT-PCR) or virus isolation. Antibodies were found in various wild and captive ruminants and some zoo animals.

Experimental infection in non-pregnant 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.,* 2012, Wernike *et al.,* 2013).

Transmission is by insect vectors and then vertically *in utero.* SBV genome was detected in several *Culicoides* species and vector competence was demonstrated. Vertical transmission across the placenta is proven but direct infection from animal to animal is very unlikely. Experimental infection was not successful via the oral route and animals that were in contact 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).

The signs in fetuses or newborns 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, cerebellar hypoplasia and enlarged thymus. The rate of malformation varies depending on the stage of gestation at the time of infection.

Serological studies in humans did not show any evidence that it is a zoonotic agent (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 NSD virus (NSDV), an *Orthonairovirus* in the *Nairoviridae* family. The disease has been identified primarily in countries of eastern Africa, where its distribution appears to be limited by the range of the tick vectors that carry NSDV. In Africa, the dominant vector is the *Ixodid* tick *Rhipicephalus appendiculatus*, although ticks of the species *Ambylomma variegatum* have also been found to carry the virus and to be competent for its transmission (Daubney & Hudson, 1934). Importantly, the virus is now known to be present in southern Asia and China (People's Rep. of). Molecular sequencing has shown that a virus previously isolated from *Haemaphysalis* ticks in India and Sri Lanka (where it was known as Ganjam virus) (Sudeep *et al.,* 2009) is also NSDV (Marczinke & Nichol, 2002), and RNA from the same virus has recently been found in *Haemaphysalis* ticks in northeast (Gong *et al.,* 2015) and central (Yang *et al.,* 2019) China. Despite this widespread distribution of the virus, there is no recorded disease in small ruminants in Asia that can be ascribed to NSDV, apart from one outbreak in imported European sheep (Ghalsasi *et al.,* 1981).

NSD in Africa is characterised by a mortality rate that 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 with sheep being more susceptible, although there are differences in susceptibility among the various breeds and strains in their response to infection with NSDV, some being more susceptible than others. Cattle and game are refractory to infection with NSDV (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 sequela to the infection. Examination of the preferred sites for the attachment of ticks, such as the ears, head and body, is likely to reveal the presence of *Rhipicephalus appendiculatus.*

Death 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, 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, 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.

NSDV is an apparently rare zoonotic agent in the field, causing a mild influenza-like disease in humans. Laboratory infection has been associated with fever and joint pains (Zeller & Bouloy, 2000).

B. DIAGNOSTIC TECHNIQUES

Table 1.1. Test methods available for the diagnosis of CVV and their purpose

[*in preparation*]

Table 1.2. Test methods available for the diagnosis of AKAV and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent						
Virus isolation	–	+	–	+	–	–
RT-PCR	–	+++	–	+	++	–
Detection of immune response						
ELISA	+++	+++	+++	+++[(a)]	+++	+++
VN	+++	+++	+++	+++[(a)]	+++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.

RT-PCR = reverse-transcription polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay; VN = virus neutralisation.
[(a)]Appropriate for confirmation of clinical cases only when fetal samples are tested prior to colostrum intake.

Table 1.3. Test methods available for the diagnosis of SBV and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent						
Virus isolation	–	+	–	+	–	–
RT-PCR	–	+++	–	+++	++	–
Detection of immune response						
ELISA	+++	+++	+++	+[(a)]	+++	+++
VN	+++	+++	+++	+[(a)]	+++	+++
IFAT	+++	+++	+++	+[(a)]	+++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay; VN = virus neutralisation; IFAT = indirect fluorescent antibody test.
[(a)]Appropriate for confirmation of clinical cases only when fetal samples are tested prior to colostrum intake.

Table 1.4. Test methods available for the diagnosis of NSD and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent						
Virus isolation in cell culture	–	–	–	++	–	–
Real-time RT-PCR	–	++	++	+++	+	–
Detection of immune response						
VN	+	–	–	++	++	++
Fluorescent staining (I)FAT)	–	–	–	+	–	+

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; VN = virus neutralisation
(I)FAT = direct or indirect fluorescent antibody test.

B1. Detection and 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.

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 RT-PCR, the Bunyamwera (BUN) and California (CAL) serogroup viruses can be distinguished. Previously described nested RT-PCR techniques allow the CAL and most of the BUN serogroup viruses to be distinguished from other *Orthobunyavirus* genus members (Kuno *et al*., 1996; Moreli *et al*., 2001). A duplex real-time RT-PCR for the CAL serogroup and CVV has been reported (Wang *et al.,* 2009), and more recently a real-time PCR that amplifies the M-segment of glycoprotein 1 of CVV and other genes of interest (Hoffmann *et al.,* 2013). However, the tests have not yet been validated for veterinary applications.

CVV antigen can be detected by immunohistochemistry in infected tissue sections using purified rabbit hyperimmune polyclonal serum against CVV propagated in Vero cells (Hoffmann *et al.,* 2012).

See Waddell *et al.* (2019) for a review and gap analysis of published CVV studies.

2. Akabane virus

Diagnosis of infection is rarely made by virus isolation, but usually by serology (ELISA, VNT) undertaken on fetal fluids, occasionally by real-time RT-PCR and sometimes by histopathology. Virus can be readily isolated from viraemic sentinel animals using plasma or buffy coat suspensions, from vector pools and occasionally from fetal material. RT-PCRs have been described for the detection of AKAV.

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

Methods employed for specific identification of AKAV using monospecific antibodies or monoclonal antibodies have included virus neutralisation (VN), and immunofluorescence (FA) (Blacksell *et al*., 1997; Gard *et al*., 1988). Antigen detection by immunoperoxidase staining can be used in formalin-fixed material of bovine and ovine fetal material, and in naturally infected newborn calves (Noda *et al*., 2001).

RT-PCR methods for the detection of AKAV nucleic acids have been developed, but additional RT-PCRs specific to Aino, Peaton and Tinaroo viruses or multiplex real-time RT-PCRs or sequencing must be performed to exclude cross reactions (Yang *et al.,* 2008; Yildirim *et al.,* 2015). Multiplex real-time RT-PCR methods (Ohasi *et al.,* 2004; Shirafuji *et al.,* 2015) have been developed for rapid, sensitive direct detection of multiple arboviruses, including AKAV.

3. Schmallenberg virus

Schmallenberg virus (SBV) can be detected by real-time RT-PCR (Bilk *et al.,* 2012; Vengust *et al.,* 2020). Several commercial PCR kits are also available. A one-step multiplex real-time RT-PCR (one-step real-time mRT-PCR) was developed for the simultaneous detection and differentiation of SBV, AKAV and AINV (Lee *et al.,* 2015) and a generic serogroup-specific RT-PCR followed by sequence analyses was described (Golender *et al.,* 2018).

Infectious virus can be isolated in cell culture. Insect cells (KC, C6/36), hamster cells (BHK), or monkey kidney cells (Vero) have been used. Samples for virus detection or isolation should be transported cooled or frozen. Serum or

EDTA (ethylenediaminetetraacetic acid) treated 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 specimens, 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 and the changes are not specific to SBV.

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.

3.1. Real-time reverse-transcription polymerase chain reaction

The method presented here targets the S-segment of SBV (Bilk *et al.,* 2012) and detects strains from different geographic locations and clinical presentations. The procedure given may require modification to accommodate individual laboratory or different RT-PCR kit requirements.

i) RNA extraction from blood, tissue samples, midges and semen

Commercial kits are widely available; the RNA extraction can be performed from the sample matrixes blood, serum, tissues and midges according to the procedures specified in each kit. For semen samples, the sensitivity depends on the application of optimized nucleic acid extraction methods combining Trizol(R) LS Reagent lysis with purification of the viral RNA with magnetic beads.

ii) Primers and probe sequences (5' → 3')

SBV-S-382F: TCA-GAT-TGT-CAT-GCC-CCT-TGC

SBV-S-469R: TTC-GGC-CCC-AGG-TGC-AAA-TC

SBV-S-408FAM: FAM-TTA-AGG-GAT-GCA-CCT-GGG-CCG-ATG-GT-BHQ1

iii) Preparation of reaction mixtures

For each PCR test, appropriate controls should be included. As a minimum, a no template control (NTC, reagents only), appropriate negative controls, e.g. 1 per 10 test samples, and a positive control should be included. The PCR amplifications are carried out in a volume of 25 µl. Prior to PCR, a master mix is made up, comprising all components except template RNA. This is then dispensed into PCR tubes or plates. Subsequently, test or control RNA (5 µl) is added to the tubes. This approach minimises pipetting errors when assaying large numbers of samples. The master mix comprises (per reaction): 12.5 µl 2 × RT-PCR buffer, 1.0 µl 25 × RT-PCR enzyme mix, 4.5 µl nuclease-free water and 2 µl SBV-specific primer-probe-mix (10 µM SBV-specific primers + 1.875 µM SBV-specific probe).

iv) Real-time reverse-transcription polymerase chain reaction

10 minutes at 45°C. 10 minutes at 95°C. 42 cycles of: 15 seconds at 95°C, 20 seconds at 55°C, and 30 seconds at 72°C. Fluorescence acquisition occurs at the 55°C step mode.

4. Nairobi sheep disease virus

Techniques for laboratory detection of NSDV have been reviewed recently (Hartlaub *et al.,* 2021). There are currently no commercial kits available for detecting the virus. The simplest method of confirming NSDV infection is one of the published methods for real-time RT-PCR (Bin Tarif *et al.*, 2012; Hartlaub *et al.*, 2021). As is the case for the other bunyaviruses, real-time RT-PCR is the standard for NSDV detection and confirmation. The live virus can also be detected using cell culture.

4.1. Real-time RT-PCR

RNA must first be purified from blood or tissue samples. Viral RNA can be detected in whole blood or serum, or in nasal or rectal swabs. The best tissues for detecting virus appear to be the spleen, liver, nasal epithelium and conjunctiva (Hartlaub *et al.*, 2021). Tissue samples should be extracted with acidified guanidine thiocyanate phenol using one of the commercial preparations available. Solid tissues (0.5–1.0 g) are minced and homogenised with 10 ml reagent. Swabs can be extracted, and whole blood, or serum homogenised with, the same reagent; RNA is then purified according to the manufacturer's instructions. For tissues, blood, serum or swabs, RNA extraction based on magnetic beads or spin columns is also suitable. The resulting RNA is stored at –70°C (or –20°C if –70°C not available) until required.

RT-PCR is based on amplification of a section of the S-segment of the virus. The published protocols use the same forward and reverse primers, and differ only in the use of SybrGreen (Bin Tarif *et al.*, 2012) or a TaqMan probe (Hartlaub *et al.*, 2021) for detection. The protocol using the TaqMan probe is given; the master mix recipe and thermal cycling conditions given are for a specific one-step RT-PCR kit, other reagents can also be used, but primer and probe concentrations and the thermocycling protocol should be optimised for other reagents.

i) Primers and probes used (5' → 3'):

Forward primer (F1):	TGA-CCA-TGC-AGA-ACC-AGA-TYG
Reverse primer (R1A):	GAA-ACA-AGC-CTC-ATG-CTA-ACC-T
Probe (P1):	FAM-CAA-GGA-TGC-CAT-CCT-TGC-ATG-GCA-BHQ1

ii) Reactions using the QuantiTect Probe RT-PCR Kit:

Add 5 µl of sample RNA to 20 µl reaction mix containing:

Reagent	Mix (1 reaction)	Final concentration
2× RT-PCR master mix	12.5 µl	
RT Mix	0.25 µl	
Probe P1 (1 µM)	0.5 µl	20 nM
Primer F1 (1 µM)	2 µl	80 nM
Primer R1A (1 µM)	2 µl	80 nM
Water	2.75 µl	
Final volume	20 µl	

iii) Thermocycling protocol

50°C for 30 minutes	1 cycle	Reverse transcription step
95°C for 15 minutes	1 cycle	Inactivates RT and activates polymerase
95°C for 15 seconds 63°C for 30 seconds 72°C for 30 seconds	48 cycles	PCR amplification of the cDNA

4.2. Virus isolation

NSDV may be isolated from material collected from field cases by the use of cell cultures (Davies *et al.*, 1977a). 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 nystatin (50 units/ml) or amphotericin B (2.5 μg/ml).

For virus isolation in cell culture, the BHK-21-Clone 13 cell line is especially valuable, but other BHK-21 clones, SW13 cells, Vero cells and primary and secondary lamb or hamster kidney cells have also been used (Shepherd *et al*., 1978). Most strains of NSDV produce a cytopathic effect (CPE) on first passage in BHK-21 cells, while others produce a more obvious CPE only after passage. CPE is not reliably produced on lamb testis or kidney cells, but may occur after multiple passages. The CPE is not specific for NSDV, which can be confirmed in RNA purified from infected cells by RT-PCR as in B1.4.1 or, in cover-slip cultures, by immunofluorescence if a specific anti-NSDV antiserum is available. If immunofluorescence is to be used for confirmation, cultures should be used both with and without flying cover-slips or microwell slide cultures should also be prepared. An appropriate volume of sample, depending on the size of the bottle, dish or well used, should be inoculated onto the cell monolayer and a period of 1–2 hours allowed for adsorption. The CPE becomes evident in BHK cell cultures as foci of granular rounded cells after 24–48 hours, and in a further 24–48 hours in other cell types. FA staining may be positive as early as 24–48 hours post-inoculation when no CPE has yet become evident. Antibodies for immunofluorescent staining may be prepared from hyperimmune mouse ascitic fluids, and from immune mouse, rabbit or sheep sera by standard methods. Such antibodies can be conjugated directly for FAT staining, or a species-specific secondary conjugate may be used (IFAT). Some cross-fluorescence may occur with other nairoviruses at low dilutions of the antibody, but these viruses are not normally associated with disease in sheep or goats.

B2. Serological tests

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

1. Cache Valley virus

1.1. Virus neutralisation test

Virus neutralisation (VN) tests for CVV used to be done by a plaque reduction neutralisation method, which may exhibit some cross-reactivity to antibodies against related BUN-serogroup viruses (Beaty *et al.,* 1989), 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 $TCID_{50}$ 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.

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 included coating of the plates with mouse ascitic fluid, followed by addition of a sucrose/acetone mouse brain antigen in a sandwich ELISA format. However, alternative methods to produce the antigen (cell culture amplified or produced by recombinant technology) should now be used in place of the mouse brain extract). 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).

2. Akabane virus

Serological assays can be used to test serum or plasma and, from stillborn or colostrum-deprived fetuses, detection of antibodies in pericardial and pleural fluids will also confirm *in utero* infection.

2.1. 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*., 1978; 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.

2.1.1. 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 1/4 to 1/128 in a 96-well flat-bottomed microtitre plate using duplicate wells and 50 µl per well. Standard controls are prepared in a similar manner.

iii) Add 50 µl per well of virus in Eagles medium diluted to provide 100 $TCID_{50}$ 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 50 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 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, 100 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.

2.2. Enzyme-linked immunosorbent assay

AKAV 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 (Ungar-Waron *et al*., 1989).

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

There are two assays commercially available as kits for use in serum and plasma samples of cattle, sheep and goats. One is a purified AKAV-based competition ELISA for detection of antibodies raised against AKAV, while the other is a purified SBV N-protein based indirect ELISA used for detection of antibodies against SBV and other Simbu serogroup viruses. The assays have different diagnostic sensitivities and

specificities (Li *et al.,* 2019; Tsuda *et al.,* 2004b). In particular, the SBV N-protein-based assay has broad reactivity with antibodies to other members of the Simbu serogroup but some cross reactivity may also be encountered with the AKAV competitive ELISA.

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; ii) indirect Immunofluorescence test; and iii) the VN test.

3.1. Enzyme-linked immunosorbent assay

Several types of ELISAs have been developed, including commercially available kits.

The systems can be divided into indirect ELISAs on the basis of recombinant N-protein, Gc-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 $TCID_{50}$/50µl (2000 $TCID_{50}$/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 prefilled 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 CO_2-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 CO_2-cabinet for incubation.

vi) Incubate the plates at 34–37°C for 3–4 days in a humidified CO_2 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 $TCID_{50}$, and the positive control serum shows the indicated titre (± one log2-step).

The antibody titre is calculated as ND_{50} according to Behrens and Kärber.

Neutralisation titre = V–d × (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. Virus neutralisation in cell culture

The titre of NSDV-neutralising antibodies may be assayed using either the inhibition of infection in 96-well microtitre plates or the reduction in plaque number in 6-well plates. BHK-21 cells and SW13 cells have been used for these assays. In each case, sera are heated at 56°C for 30 minutes before use to inactivate complement.

4.1.1. Infection inhibition in microtitre plates

i) The procedure requires a stock of NSDV with a known titre (determined on the cell line to be used for the neutralisation assay) and a stock of those cells, trypsinised into suspension and adjusted to 2×10^5/ml (BHK-21) or 10^5/ml (SW13) (5ml of cells per 96-well plate).

ii) Each serum dilution is assayed in at least four replicate wells. Two-fold serial dilutions of serum (starting from 1/5 or 1/10, depending on the expected titre) are prepared in flat-bottomed 96-well microtitre plates (50 µl/well).

iii) Add 50 µl maintenance medium containing approximately 100 $TCID_{50}$ of NSDV, mix well by pipetting up and down, cover the plate and incubate for 1 hour at 37°C.

iv) Titrate the stock of diluted virus to ensure that the concentration is in the range 90–150 $TCID_{50}$ per 50 µl.

v) Add 50 µl of cell suspension per well, mix by tapping the sides of the plate, and incubate the plates at 37°C, 5% CO_2.

vi) The wells are scored directly for CPE at 5 dpi (BHK-21 cells) or fixed with neutral buffered formalin at 7 dpi and stained with 0.1% crystal violet to visualise lesions (SW13 cells).

vii) The neutralising titre is the serum dilution that inhibits infection in 50% of the replicate wells (ND_{50}), which can be calculated using the Spearman-Kärber equation. If X1 is the greatest dilution where no well shows CPE and ΣP is the sum of the proportions of uninfected wells from row X1 onwards, the ND_{50} is 10^m where m = (log[X1] – log[2] × [ΣP-0.5]). E.g. for X1=1/40 and ΣP=1.75, m = -1.6 – (0.301 × 1.25) = -1.97625, and ND_{50} = 1/94.68.

4.1.2. Plaque reduction

i) SW13 cells are plated in 6-well plates and should be sub-confluent when used.

ii) Two-fold dilutions of serum (600 µl) are mixed with approx. 50 $TCID_{50}$ of NSDV in 600 µl maintenance medium and incubated for 1 hour at 37°C.

iii) The medium is removed from cells in six-well plates and replaced with 500 µl aliquots of this mixture (in duplicate). The plates are incubated for 1 hour at 37°C, with gentle rocking every 15 minutes. Control wells are infected with virus that has been incubated without serum.

iv) Prepare an overlay by mixing equal volumes of an autoclaved solution of 1.8% Bacto-Agar and 2× MEM containing 4% FCS and 2× penicillin/streptomycin, and keep at ~40°C to prevent it solidifying.

v) The inocula are removed and cells overlaid with 2 ml per well Bacto-Agar/MEM, the overlay is allowed to set at room temperature (15–20 minutes) and then the plates are incubated at 37˚C in a CO_2 incubator.

vi) After 4 days, a second overlay consisting of 0.1% crystal violet in neutral buffered formalin is added. After a further 24 hours, the overlays are removed and plaques counted visually. The mean number of plaques in the control wells is set as 100%, and the neutralising titre is the greatest serum dilution that still reduces the plaque number by 80% ($PRNT_{80}$).

4.2. Indirect fluorescent antibody test

The indirect fluorescent antibody test (IFAT) can also be used 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 NSDV 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. The NSDV I-34 strain[2] was the virus used to prepare antigen in the original studies, a strain that has been adapted to grow in BHK-21-Clone 13 cells, after a series of passages. Many other strains of NSDV have been found to grow in these cells, in BSR-T7 cells (a BHK cell derivative) or in Vero cells. CPE is greatly reduced in Vero cells, and this can be an advantage in the preparation of antigen (infected cell monolayers) for this assay.

The virus antigen in the cell substrate of choice may be prepared for the test on loose coverslips, on chambered multiwell slides, PTFE(Teflon)-printed multiwell slides or in flat-bottomed microtitre plates. If using loose coverslips, the 9 mm diameter size are suitable for 12-well multiwell plates. A method using PTFE-printed multiwell slides is described.

4.2.1. Preparation of antigen slides

i) Wash and sterilise the slides. This is done briefly with a hot detergent that is used for tissue culture glassware in the laboratory, followed by three rinses in tap water for 30 minutes, each followed by similar rinses in distilled/deionised water. Slides are then placed in 70% ethanol for 10 minutes, removed with 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 sterile forceps.

iii) Prepare a suspension of BHK cells containing approximately 25,000 cells/ml in growth medium, and add 1000 $TCID_{50}$ of NSDV per ml. Mix by pipetting.

iv) Add the infected cells in 50 µl volumes (for the 12-well size) or as appropriate to the size of the wells on the PTFE slides. Replace the cover on the dishes and put into a humidified CO_2 incubator. Prepare negative control slides in the same way using uninfected cells.

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; individual laboratories should determine the optimal harvesting time for their cells and virus strain by fixing and staining slides after 24, 36 and 48 hours).

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 14733 – 00800 Nairobi, Kenya.

vii) The slides are washed three times in PBS and the cells fixed. This can be done using dry heat (minimum 80°C) or with ice-cold acetone or methanol:acetone (1:1) for 10 minutes. After fixing in acetone or methanol:acetone, wash the slides three times (5 minutes per wash) in PBS and then allow to air dry. 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 loose coverslips or in multiwell plates or chambered multiwell slides. When using plastic tissue culture multiwell plates, however, fixation should not be with 100% acetone.

4.2.2. Test procedure

i) Hydrate the slides by adding a drop of PBS to each well with a Pasteur pipette. Number the slides according to the number of sera to be tested; at least 12 slides are needed per serum. Include positive and negative control sera, with infected and uninfected cell cultures, in every assay.

ii) Prepare a 2-fold dilution series for each serum, starting at 1/80 and finishing at 1/2560 (6 dilutions)

iii) Discard the PBS and add the serum dilutions in a predetermined manner to the wells. It is preferable to duplicate each dilution, on the same or another slide.

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

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

vi) Add the fluorescein-conjugated anti-species conjugate (usually anti-sheep or anti-goat) at a predetermined working dilution (must be determined in each laboratory depending on the conjugated antibody used); one drop can be added to each well with a Pasteur or other pipette.

vii) Incubate as before for 30 minutes.

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

ix) Examine the slides by fluorescent microscopy. NSDV 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 uninfected control cells.

x) Sera that show this fluorescence at dilutions of 1/640 or 1/1280 are indicative of recent infection with NSDV (Davies *et al*., 1976).

4.3. Other tests

An ELISA using a partially purified tissue culture antigen has been described for antibody testing and is suitable for use in serological surveys (Hartlaub *et al.,* 2021). Virus neutralisation or the IFA test should, however, be used to check doubtful results (Munz *et al*., 1984). Furthermore, new ELISAs are under evaluation (Hartlaub *et al.,* 2021).

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

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 leukopoenia, 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 (Kim *et al.,* 2011).

4. Schmallenberg virus

Three inactivated commercial vaccines to protect sheep and cattle from SBV infection have received regulatory approval in Europe. Several other forms of vaccines (recombinant modified live, vector, subunit vaccines) have been developed and are at different levels of evaluation (Wernike & Beer*,* 2020).

Most trials showed a high efficacy and safety of the different vaccine approaches to protect against SBV.

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 Schmallenberg virus,
refer to the Friedrich-Loeffler-Institut, Insel Riems, Germany.

NB: FIRST ADOPTED IN 2004 AS BUNYAVIRAL DISEASES OF ANIMALS (EXCLUDING RIFT VALLEY FEVER);
MOST RECENT UPDATES ADOPTED IN 2023.