

CHAPTER 2.4.13.

LUMPY SKIN DISEASE

SUMMARY

Description of the disease: Lumpy skin disease (LSD, knopvelsiekte) is a pox disease of cattle characterised by fever, nodules on the skin, mucous membranes and internal organs, emaciation, enlarged lymph nodes, oedema of the skin, and sometimes death. The disease is of economic importance as it can cause a temporary reduction in milk production, temporary or permanent sterility in bulls, damage to hides and death due to secondary bacterial infections. Various strains of capripoxvirus are responsible for the disease. These are antigenically indistinguishable from strains causing sheep pox and goat pox yet distinct at the genetic level. LSD has a partially different geographical distribution from sheep and goat pox, suggesting that cattle strains of capripoxvirus do not infect and transmit between sheep and goats. Transmission of LSD virus (LSDV) is thought to be predominantly by insects, natural contact transmission in the absence of insect vectors being inefficient. Lumpy skin disease occurs in most African countries. In 2015, outbreaks outside of Africa have occurred in the Middle East, Turkey, Greece, Russia and the Republic of Georgia.

Identification of the agent: Laboratory confirmation of LSD is most rapid using a polymerase chain reaction (PCR) method specific for capripoxviruses or by the demonstration of typical capripox virions in biopsy material or desiccated crusts using the transmission electron microscope in combination with a clinical history of a generalised nodular skin disease and enlarged superficial lymph glands in cattle. Capripoxvirus is distinct from parapoxvirus, which causes bovine papular stomatitis and pseudocowpox, but cannot be distinguished morphologically from cowpox and vaccinia virus, both orthopoxvirus infections of cattle. Neither of these, however, causes generalised infection and both are uncommon in cattle. LSDV will grow in tissue culture of bovine, ovine or caprine origin, although maximum yield is obtained using lamb testis or bovine dermis cells. Capripoxvirus causes a characteristic cytopathic effect and intracytoplasmic inclusion bodies, and is distinct from the virus of pseudo-LSD (Allerton – herpes mammilitis), which is a herpesvirus producing syncytia and intranuclear inclusion bodies. The antigen of capripoxvirus can be demonstrated in tissue culture using immunoperoxidase or immunofluorescent staining and the virus can be neutralised using specific antisera.

An antigen-detection enzyme-linked immunosorbent assay (ELISA) using a polyclonal detection serum raised against a recombinant immunodominant antigen of capripoxvirus and an antibody-detecting ELISA based on the purified whole virus have been described. A variety of conventional and real-time PCR tests as well as isothermal amplification tests using capripoxvirus-specific primers have been published for use on ocular swabs, blood, tissue and semen samples.

Serological tests: The virus neutralisation test is the most specific serological test, but the test is not sufficiently sensitive to identify animals that have had contact with LSDV and developed only low levels of neutralising antibody. The agar gel immunodiffusion test and indirect immunofluorescent antibody test are less specific due to cross-reactions with antibody to other poxviruses. Western blotting using the reaction between the P32 antigen of LSDV with test sera is both sensitive and specific, but is difficult and expensive to carry out. The use of this or another appropriate antigen, expressed by a suitable vector, in an ELISA offers the prospect of an acceptable and standardised serological test.

Requirements for vaccines: All strains of capripoxvirus examined so far, whether derived from cattle, sheep or goats, share immunising antigens. Attenuated cattle strains, and strains derived from sheep and goats have been used as live vaccines.

A. INTRODUCTION

Lumpy skin disease (LSD) was first seen in Zambia in 1929, spreading into Botswana by 1943 (Haig, 1957), and then into South Africa, where it affected over eight million cattle causing major economic loss. In 1957 it entered Kenya, associated with an outbreak of sheep pox (Weiss, 1968). In 1970 LSD spread north into the Sudan, by 1974 it had spread west as far as Nigeria, and in 1977 was reported from Mauritania, Mali, Ghana and Liberia. Another epizootic of LSD between 1981 and 1986 affected Tanzania, Kenya, Zimbabwe, Somalia and the Cameroon, with reported mortality rates in affected cattle of 20%. The occurrence of LSD north of the Sahara desert and outside the African continent was confirmed for the first time in Egypt and Israel between 1988 and 1989, and after a period of 17 years was reported again in 2006 (Brenner *et al.*, 2006). In the past decade, LSD occurrences have been reported in the Middle Eastern, European and west Asian regions (OIE, 2015). Lumpy skin disease outbreaks tend to be sporadic, depending upon animal movements, immune status, and wind and rainfall patterns affecting vector populations. LSD is considered to have become established outside Africa. The principal method of transmission is thought to be mechanical by arthropod vector (Tuppurainen *et al.*, 2015).

The severity of clinical signs of LSD depends on the strain of capripoxvirus and the age, immunological status and breed of host. *Bos taurus* is more susceptible to clinical disease than *Bos indicus*; the Asian buffalo has also been reported to be susceptible. Within *Bos taurus*, the fine-skinned Channel Island breeds develop more severe disease, with lactating cows appearing to be the most at risk. However, even among groups of cattle of the same breed kept together under the same conditions, there is a large variation in the clinical signs presented, ranging from subclinical infection to death (Carn & Kitching, 1995). There may be failure of the virus to infect the whole group, probably depending on the virulence of the virus isolate, immunological status of the host and vector prevalence.

The incubation period under field conditions has not been reported, but following inoculation is 6–9 days until the onset of fever. In the acutely infected animal, there is an initial pyrexia, which may exceed 41°C and persist for 1 week. All the superficial lymph nodes become enlarged. In lactating cattle there is a marked reduction in milk yield. In severe cases, nodules of 2–5 cm in diameter develop over the body, particularly on the head, neck, udder and perineum between 7 and 19 days after virus inoculation (Coetzer, 2004). These nodules involve the dermis and epidermis and may initially exude serum, but over the following 2 weeks necrotic plugs may appear penetrating the full thickness of the hide. At the appearance of clinical signs, the discharge from the eyes and nose becomes mucopurulent, and keratitis may develop. Nodules may also develop in the mucous membranes of the mouth and alimentary tract, particularly the abomasum and in the trachea and the lungs, resulting in primary and secondary pneumonia. The nodules on the mucous membranes of the eyes, nose, mouth, rectum, udder and genitalia quickly ulcerate, and by then all secretions, ocular and nasal discharge and saliva contain LSD virus (LSDV). The limbs may be oedematous and the animal is reluctant to move. Pregnant cattle may abort, and there are reports of intrauterine transmission (Roubya & Aboulsoudb, 2016). Bulls may become permanently or temporarily infertile and the virus can be excreted in the semen for prolonged periods (Irons *et al.*, 2005). Recovery from severe infection is slow; the animal is emaciated, may have pneumonia and mastitis, and the necrotic plugs of skin, which may have been subject to fly strike, are shed leaving deep holes in the hide (Prozesky & Barnard, 1982).

LSDV is not transmissible to humans.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of LSD 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
Agent identification¹						
Virus isolation	+	++	+	+++	+	n/a
Antigen detection	++	++	++	++	++	n/a

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
PCR	++	+++	++	+++	++	n/a
Detection of immune response						
VN	++	++	++	++	++	++
IFAT	+	+	+	+	+	+

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose; n/a = not applicable. Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

PCR = polymerase chain reaction; VN = virus neutralisation; IFAT = indirect fluorescent antibody test.

1. A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agent

1.1. Sample collection, submission and preparation

Material for virus isolation and antigen detection should be collected by biopsy or at post-mortem from skin nodules, lung lesions or lymph nodes. Samples for virus isolation and antigen-detection enzyme-linked immunosorbent assay (ELISA) should be collected within the first week of the occurrence of clinical signs, before the development of neutralising antibodies (Davies, 1991; Davies *et al.*, 1971). Samples for genome detection by polymerase chain reaction (PCR) may be collected when neutralising antibody is present. Following the first appearance of the skin lesions, the virus can be isolated for up to 35 days and viral nucleic acid can be demonstrated by PCR for up to 3 months (Tuppurainen *et al.*, 2005; Weiss, 1968). Buffy coat from blood collected into heparin or EDTA (ethylene diamine tetra-acetic acid) during the viraemic stage of LSD (before generalisation of lesions or within 4 days of generalisation), can also be used for virus isolation. Samples for histology should include tissue from the surrounding area and should be placed immediately following collection into ten times the sample volume of 10% formalin.

Tissues in formalin have no special transportation requirements. Blood samples with anticoagulant for virus isolation from the buffy coat should be placed immediately on ice and processed as soon as possible. In practice, the samples may be kept at 4°C for up to 2 days prior to processing, but should not be frozen or kept at ambient temperatures. Tissues for virus isolation and antigen detection should be kept at 4°C, on ice or at –20°C. If it is necessary to transport samples over long distances without refrigeration, the medium should contain 10% glycerol; the samples should be of sufficient size, e.g. 1/10 (g/ml), that the transport medium does not penetrate the central part of the biopsy, which should be used for virus isolation.

Material for histology should be prepared by standard techniques and stained with haematoxylin and eosin (H&E) (Burdin, 1959). Lesion material for virus isolation and antigen detection is minced using sterile scalpel blade and forceps and then ground with a pestle in a sterile mortar with sterile sand and an equal volume of sterile phosphate buffered saline (PBS) containing sodium penicillin (1000 international units [IU]/ml), streptomycin sulphate (1 mg/ml), mycostatin (100 IU/ml) or fungizone (amphotericin, 2.5 µg/ml) and neomycin (200 IU/ml). The suspension is freeze-thawed three times and then partially clarified by centrifugation using a bench centrifuge at 600 *g* for 10 minutes. In cases where bacterial contamination of the sample is expected (such as when virus is isolated from skin samples), the supernatant can be filtered through a 0.45 µm pore size filter after the centrifugation step. Buffy coats may be prepared from unclotted blood by centrifugation at 600 *g* for 15 minutes, and the buffy coat carefully removed into 5 ml of cold double-distilled water using a sterile Pasteur pipette. After 30 seconds, 5 ml of cold double-strength growth medium is added and mixed. The mixture is centrifuged at 600 *g* for 15 minutes, the supernatant is discarded and the cell pellet is suspended in 5 ml growth medium, such as Glasgow's modified Eagle's medium (GMEM). After centrifugation at 600 *g* for a further 15 minutes, the resulting pellet is suspended in 5 ml of fresh GMEM. Alternatively, the buffy coat may be separated from a heparinised sample by using a Ficoll gradient.

1.2. Virus isolation on cell culture

LSDV will grow in tissue culture of bovine, ovine or caprine origin, although primary or secondary culture of bovine dermis cells or lamb testis (LT) cells are considered to be the most susceptible, particularly those derived from a breed of wool sheep. One ml of clarified supernatant or buffy coat, is inoculated on to a 25 cm² culture flask at 37°C and allowed to adsorb for 1 hour. The culture is then washed with warm PBS and covered with 10 ml of a suitable medium, such as GMEM, containing antibiotics and 2% fetal calf serum. If available, tissue culture tubes containing LT cells and a flying cover-slip, or tissue culture microscope slides, are also infected.

The flasks are examined daily for 7–14 days for evidence of cytopathic effect (CPE). Infected cells develop a characteristic CPE consisting of retraction of the cell membrane from surrounding cells, and eventually rounding of cells and margination of the nuclear chromatin. At first only small areas of CPE can be seen, sometimes as soon as 2 days after infection; over the following 4–6 days these expand to involve the whole cell sheet. If no CPE is apparent by day 14, the culture should be freeze-thawed three times, and clarified supernatant inoculated on to fresh LT culture. At the first sign of CPE in the flasks, or earlier if a number of infected cover-slips are being used, a cover-slip should be removed, fixed in acetone and stained using H&E. Eosinophilic intracytoplasmic inclusion bodies, which are variable in size but up to half the size of the nucleus and surrounded by a clear halo, are diagnostic for poxvirus infection. PCR may be used as an alternative to H&E for confirmation of the diagnosis. The CPE can be prevented or delayed by adding specific anti-LSD serum to the medium. In contrast, the herpesvirus that causes pseudo-LSD produces a Cowdry type A intranuclear inclusion body. It also forms syncytia, which are not usually a feature of capripoxvirus infection (although they may be seen in Madin–Darby bovine kidney [MDBK] cells).

Strains of capripoxvirus that cause LSD have been adapted to grow on the chorioallantoic membrane of embryonated chicken eggs and African green monkey kidney (Vero) cells. This is not recommended for primary isolation. Ovine testis secondary cell line (OA3.Ts) has been evaluated for the propagation of capripoxvirus isolates (Babiuk *et al.*, 2007).

1.3. Electron microscopy

The characteristic poxvirus virion can be visualised using a negative staining preparation technique followed by examination with an electron microscope. There are many different negative staining protocols, an example is given below.

Before centrifugation, material from the original biopsy suspension is prepared for examination under the transmission electron microscope by floating a 400-mesh hexagon electron microscope grid, with pioloform-carbon substrate activated by glow discharge in pentylamine vapour, on to a drop of the suspension placed on parafilm or a wax plate. After 1 minute, the grid is transferred to a drop of Tris/EDTA buffer, pH 7.8, for 20 seconds and then to a drop of 1% phosphotungstic acid, pH 7.2, for 10 seconds. The grid is drained using filter paper, air-dried and placed in the electron microscope. The capripox virion is brick shaped, covered in short tubular elements and measures approximately 290 × 270 nm. A host-cell-derived membrane may surround some of the virions, and as many as possible should be examined to confirm their appearance (Kitching & Smale, 1986).

The virions of capripoxvirus are indistinguishable from those of orthopoxvirus, but, apart from Vaccinia virus and Cowpox virus, which are both uncommon in cattle and do not cause generalised infection, no other orthopoxvirus causes lesions in cattle. However, Vaccinia virus may cause generalised infection in young immunocompromised calves. In contrast, orthopoxviruses are a common cause of skin disease in domestic buffalo causing buffalo pox, a disease that usually manifests as pock lesions on the teats, but may cause skin lesions at other sites, such as the perineum, the medial aspects of the thighs and the head. Orthopoxviruses that cause buffalo pox cannot be readily distinguished from capripoxvirus by electron microscopy. The virions of parapoxvirus that cause bovine papular stomatitis and pseudocowpox are smaller, oval in shape and each is covered in a single continuous tubular element that appears as striations over the virion. The capripoxvirus is also distinct from the herpesvirus that causes pseudo-LSD (Allerton – herpes mammillitis).

1.4. Fluorescent antibody tests

Capripoxvirus antigen can also be identified on the infected cover-slips or tissue culture slides using fluorescent antibody tests. Cover-slips or slides should be washed and air-dried and fixed in cold acetone for 10 minutes. The indirect test using immune cattle sera is subject to high background colour and nonspecific reactions. However, a direct conjugate can be prepared from sera from convalescent cattle (or from sheep or goats convalescing from capripox) or from rabbits hyperimmunised with

purified capripoxvirus. Uninfected tissue culture should be included as a negative control as cross-reactions can cause problems due to antibodies to cellular components.

1.5. Polymerase chain reaction (PCR)

The conventional gel-based PCR method described below is a simple, fast and sensitive method for the detection of capripoxvirus genome in EDTA blood, biopsy, semen or tissue culture samples. More recently, quantitative real-time PCR methods that are reported to be faster, with higher sensitivity, have been described (Balinsky *et al.*, 2008; Bowden *et al.*, 2008). These PCR-based protocols do not allow differentiation between LSD and sheep and goat pox viruses. Sequence and phylogenetic analysis of particular genomic regions are required to differentiate the three species of capripoxvirus (Le Goff *et al.*, 2009). Previously, virus isolates were characterised by comparing the genome fragments generated by *Hind*III digestion of their purified DNA (Black *et al.*, 1986; Kitching *et al.*, 1989), however this technique lacked robustness, possibly due to recombination between virus strains (Gershon & Black, 1987; Gershon *et al.*, 1989).

An example of a published conventional gel-based PCR method is described below (Tuppurainen *et al.*, 2005).

1.5.1. Test procedure

The extraction method described below can be replaced using commercially available DNA extraction kits.

- i) Freeze and thaw 200 µl of blood in EDTA, semen or tissue culture supernatant and suspend in 100 µl of lysis buffer containing 5 M guanidine thiocyanate, 50 mM potassium chloride, 10 mM Tris/HCl (pH 8); and 0.5 ml Tween 20.
- ii) Cut skin and other tissue samples into fine pieces using sterile scalpel blade and forceps. Grind with a pestle in a mortar. Suspend the tissue samples in 800 µl of the above mentioned lysis buffer.
- iii) Add 2 µl of proteinase K (20 mg/ml) to blood samples and 10 µl of proteinase K to tissue samples. Incubate at 56°C for 2 hours or overnight, followed by heating at 100°C for 10 minutes. Add phenol:chloroform:isoamylalcohol (25:24:1 [v/v]) to the samples in 1:1 ratio. Vortex and incubate at room temperature for 10 minutes. Centrifuge the samples at 16,060 *g* for 15 minutes at 4°C. Carefully collect the upper, aqueous phase (up to 200 µl) and transfer into a clean 2.0 ml tube. Add two volumes of ice cold ethanol (100%) and 1/10 of 3 M sodium acetate (pH 5.3). Place the samples at –20°C for 1 hour. Centrifuge again at 16,060 *g* for 15 minutes at 4°C and discard the supernatant. Wash the pellets with ice cold 70% ethanol (100 µl) and centrifuge at 16,060 *g* for 1 minute at 4°C. Discard the supernatant and dry the pellets thoroughly. Suspend the pellets in 30 µl of nuclease-free water and store immediately at –20°C (Tuppurainen *et al.*, 2005).
- iv) The primers were developed from the viral attachment protein encoding gene. The size of the expected amplicon is 192 bp (Ireland & Binepal, 1998). The primers have the following gene sequences:

Forward primer 5'-TCC-GAG-CTC-TTT-CCT-GAT-TTT-TCT-TAC-TAT-3'

Reverse primer 5'-TAT-GGT-ACC-TAA-ATT-ATA-TAC-GTA-AAT-AAC-3'.

- v) DNA amplification is carried out in a final volume of 50 µl containing: 5 µl of 10 × PCR buffer, 1.5 µl of MgCl₂ (50 mM), 1 µl of dNTP (10 mM), 1 µl of forward primer, 1 µl of reverse primer, 1 µl of DNA template (~10 ng), 0.5 µl of *Taq* DNA polymerase and 39 µl of nuclease-free water. The volume of DNA template required may vary and the volume of nuclease-free water must be adjusted to the final volume of 50 µl.
- vi) Run the samples in a thermal cycler: first cycle: 2 minutes at 95°C, second cycle: 45 seconds at 95°C, 50 seconds at 50°C and 1 minute at 72°C. Repeat the second cycle 34 times. Last cycle: 2 minutes at 72°C and hold at 4°C until analysis.
- vii) Mix 10 µl of each sample with loading dye and load onto a 1.5% agarose gel in TAE buffer (Tris/acetate buffer containing EDTA). Load a parallel lane with a 100 bp DNA-marker ladder. Electrophoretically separate the products approximately 8–10 V/cm for 40–60 minutes and visualise.

1.6. Isothermal genome amplification

Molecular tests using loop-mediated isothermal amplification to detect capripoxvirus genomes are reported to provide sensitivity and specificity similar to real-time PCR with a simpler method and lower cost (Das *et al.*, 2012; Murray *et al.*, 2013).

2. Serological tests

All the viruses in the *Capripoxvirus* genus share a common major antigen for neutralising antibodies and it is thus not possible to distinguish strains of capripoxvirus from cattle, sheep or goats using serological techniques.

2.1. Virus neutralisation

A test serum can either be titrated against a constant titre of capripoxvirus (100 TCID₅₀ [50% tissue culture infective dose]) or a standard virus strain can be titrated against a constant dilution of test serum in order to calculate a neutralisation index. Because of the variable sensitivity of tissue culture to capripoxvirus, and the consequent difficulty of ensuring the use of 100 TCID₅₀, the neutralisation index is the preferred method although it does require a larger volume of test sera. The test is described using 96-well flat-bottomed tissue-culture grade microtitre plates, but it can be performed equally well in tissue culture tubes with the appropriate changes to the volumes used, although it is more difficult to read an end-point in tubes. The use of Vero cells in the virus neutralisation test has been reported to give more consistent results.

2.1.1. Test procedure

- i) Test sera including a negative and a positive control are diluted 1/5 in Eagle's/HEPES (N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid) and inactivated at 56°C for 30 minutes.
- ii) Next, 50 µl of the first inactivated serum is added to columns 1 and 2, rows A to H of the microtitre plate. The second serum is placed in columns 3 and 4, the third in columns 5 and 6, the positive control serum is placed in columns 7 and 8, the negative control serum is placed in columns 9 and 10, and 50 µl of Eagle's/HEPES without serum is placed in columns 11 and 12 and to all wells in row H.
- iii) A reference strain of capripoxvirus, usually a vaccine strain known to grow well in tissue culture, with a titre of over log₁₀ 6 TCID₅₀ per ml is diluted in Eagle's/HEPES in bijoux bottles to give a log dilution series of log₁₀ 5.0; 4.0; 3.5; 3.0; 2.5; 2.0; 1.5 TCID₅₀ per ml (equivalent to log₁₀ 3.7; 2.7; 2.2; 1.7; 1.2; 0.7; 0.2 TCID₅₀ per 50 µl).
- iv) Starting with row G and the most diluted virus preparation, 50 µl of virus is added to each well in that row. This is repeated with each virus dilution, the highest titre virus dilution being placed in row A.
- v) The plates are covered and incubated for 1 hour at 37°C.
- vi) LT cells are prepared from pregrown monolayers as a suspension of 10⁵ cells/ml in Eagle's medium containing antibiotics and 2% fetal calf serum. Following incubation of the microtitre plates, 100 µl of cell suspension is added to all the wells, except wells H11 and H12, which serve as control wells for the medium. The remaining wells of row H are cell and serum controls.
- vii) The microtitre plates are covered and incubated at 37°C for 9 days.
- viii) Using an inverted microscope, the monolayers are examined daily from day 4 for evidence of CPE. There should be no CPE in the cells of row H. Using the 0240 KSGP vaccine strain of capripoxvirus, the final reading is taken on day 9, and the titre of virus in each duplicate titration is calculated by the Kärber method. If left longer, there is invariably a 'breakthrough' of virus in which virus that was at first neutralised appears to disassociate from the antibody.
- ix) *Interpretation of the results:* The neutralisation index is the log titre difference between the titre of the virus in the negative serum and in the test serum. An index of ≥1.5 is positive. The test can be made more sensitive if serum from the same animal is examined before and after infection. Because the immunity to capripox is predominantly cell mediated, a negative result, particularly following vaccination in which the response is necessarily mild, does not imply that the animal from which the serum was taken is not protected.

A constant-virus/varying-serum method has been described using serum dilutions in the range 1/5 to 1/500 and fetal calf muscle cells. Because these cells have a lower sensitivity to capripoxvirus than LT cells, the problem of virus 'breakthrough' is overcome.

Antibodies to capripoxvirus can be detected from day 2 after the onset of clinical signs. These remain detectable for about 7 months, but a significant rise in titre is usually seen between days 21 and 42.

2.2. Indirect fluorescent antibody test

Capripoxvirus-infected tissue culture grown on flying cover-slips or tissue culture microscope slides can be used for the indirect fluorescent antibody test. Uninfected tissue culture control, and positive and negative control sera, should be included in the test. The infected and control cultures are fixed in acetone at -20°C for 10 minutes and stored at 4°C . Dilutions of test sera are made in PBS, starting at 1/20 or 1/40, and positives are identified using an anti-bovine gamma-globulin conjugated with fluorescein isothiocyanate. Antibody titres may exceed 1/1000 after infection. Sera may be screened at 1/50 and 1/500. Cross-reactions can occur with orf (contagious pustular dermatitis of sheep virus), bovine papular stomatitis and perhaps other poxviruses.

2.3. Western blot analysis

Western blotting of test sera against capripoxvirus-infected cell lysate provides a sensitive and specific system for the detection of antibody to capripoxvirus structural proteins, although the test is expensive and difficult to carry out.

Capripoxvirus-infected LT cells should be harvested when 90% CPE is seen, freeze-thawed three times, and the cellular debris pelleted by centrifugation. The supernatant should be decanted, and the proteins should be separated by SDS/PAGE (sodium dodecyl sulphate/polyacrylamide gel electrophoresis). A vertical discontinuous gel system, using a stacking gel made up of acrylamide 5% in Tris (125 mM), pH 6.8, and SDS (0.1%), and a resolving gel made up of acrylamide (10–12.5%) in Tris (560 mM), pH 8.7, and SDS (0.1%), is recommended for use with a glycine running buffer containing Tris (250 mM), glycine (2 M), and SDS (0.1%). Samples of supernatant should be prepared by boiling for 5 minutes with an appropriate lysis buffer prior to loading. Alternatively, purified virus or recombinant antigens may replace tissue-culture-derived antigen.

Molecular weight markers should be run concurrently with the protein samples. The separated proteins in the SDS/PAGE gel should be transferred electrophoretically to a nitrocellulose membrane (NCM). After transfer, the NCM is rinsed thoroughly in PBS and blocked in 3% bovine serum albumin (BSA) in PBS, or 5% skimmed milk powder in PBS, on a rotating shaker at 4°C overnight. The NCM can then be separated into strips by employing a commercial apparatus to allow the concurrent testing of multiple serum samples, or may be cut into strips and each strip incubated separately thereafter. The NCM is washed thoroughly with five changes of PBS for 5 minutes on a rotating shaker, and then incubated at room temperature on the shaker for 1.5 hours, with the appropriate serum at a dilution of 1/50 in blocking buffer (3% BSA and 0.05% Tween 20 in PBS; or 5% milk powder and 0.05% Tween 20 in PBS). The membrane is again thoroughly washed and incubated (in blocking buffer) with anti-species immunoglobulin horseradish-peroxidase-conjugated immunoglobulins at a dilution determined by titration. After further incubation at room temperature for 1.5 hours, the membrane is washed and a solution of diaminobenzidintetrahydrochloride (10 mg in 50 ml of 50 mM Tris/HCl, pH 7.5, and 20 μl of 30% [v/v] hydrogen peroxide) is added. This is then incubated for approximately 3–7 minutes at room temperature on a shaker with constant observation, and the reaction is stopped by washing in PBS before excessive background colour is seen. A positive and negative control serum should be used on each occasion.

Positive test samples and the positive control will produce a pattern consistent with reaction to proteins of molecular weights 67, 32, 26, 19 and 17 kDa – the major structural proteins of capripoxvirus – whereas negative serum samples will not react with this pattern. Hyperimmune serum prepared against parapoxvirus (bovine papular stomatitis, pseudocowpox) will react with some of the capripoxvirus proteins, but not the 32 kDa protein that is specific for capripoxvirus.

2.4. Enzyme-linked immunosorbent assay

Attempts to develop an ELISA for the detection of capripoxviral antibodies have been made but the technique is not currently recommended for use. The ELISAs used as antigen expressed structural P32 protein (vaccinia H3L homologue), whole heat-inactivated sheep pox virus, capripoxvirus virion core proteins encoded by 2 ORF and expressed in *Escherichia coli*, and synthetic peptide targeting the major antigen P32 (Babiuk *et al.*, 2009; Bowden *et al.*, 2009; Heine *et al.*, 1999; Tian *et al.*, 2010). Lack of success with the assays was attributable to factors such as difficulties with the production of

inactivated whole virus in sufficient volumes, instability of recombinant antigens, low sensitivity and specificity or lack of cattle sera to use for optimisation and validation (Tuppurainen & Oura, 2012).

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Four live attenuated strains of capripoxvirus have been used as vaccines specifically for the control of LSD (Brenner *et al.*, 2006; Capstick & Coakley, 1961; Carn, 1993): a strain of Kenyan LSDV passaged 18 times in LT or fetal calf muscle cells and previously thought to be sheep and goat pox virus (Tuppurainen *et al.*, 2014), Yugoslavian RM 65 sheep pox strain, Romanian sheep pox strain and lumpy skin disease virus strain from South Africa, passaged 60 times in lamb kidney cells and 20 times on the chorioallantoic membrane of embryonated chicken eggs. All strains of capripoxvirus examined so far, whether of bovine, ovine or caprine origin, share a major neutralising site, so that animals recovered from infection with one strain are resistant to infection with any other strain. Consequently, it is possible to protect cattle against LSD using strains of capripoxvirus derived from sheep or goats (Coakley & Capstick, 1961). In 1989 and 1990 the Romanian strain of sheep pox vaccine was used to help control the LSD outbreak in Egypt (Michael *et al.*, 1996). However, it is essential to carry out controlled trials, particularly using the most susceptible breeds in peak lactation, prior to introducing a vaccine strain not usually used in cattle. The duration of protection provided by LSD vaccination is unknown. Capripoxvirus vaccine strains can produce a large local reaction at the site of inoculation in *Bos taurus* breeds (Davies, 1991), which some stock owners find unacceptable. This has discouraged the use of vaccine.

2. Outline of production and minimum requirements for conventional vaccines

General requirements set for the facilities used for the production of vaccines and for the documentation and record keeping throughout the whole manufacturing process are described in Chapter 1.1.8 *Principles of veterinary vaccine production*. The documentation should include standard operating procedures (SOP) for the method of manufacture and each step for the testing of cells and reagents used in the process, each batch and the final product.

2.1. Characteristics of the seed

2.1.1. Biological characteristics

Each seed strain of capripoxvirus used for vaccine production must be accompanied by records clearly and accurately describing its origin, isolation and tissue culture or animal passage history.

A quantity of master seed vaccine virus should be prepared, frozen or desiccated and stored at low temperatures such as -40°C or -80°C in order to provide a consistent working seed for regular vaccine production. The virus should be cultured in primary or secondary LT cells of wool sheep origin for maximum yield. Vero cells may also be used.

Each seed strain must be safe to use in all breeds of cattle for which it is intended, including young and pregnant animals. It must also be non-transmissible, remain attenuated after further tissue culture passage, and provide complete protection against challenge with virulent field strains for a minimum of 1 year. It must produce a minimum clinical reaction in all breeds of cattle when given by the recommended route.

The necessary safety and potency tests are described in Section C.2.2.4 *Final product batch tests*.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Each master seed must be tested to ensure its identity and shown to be free from adventitious viruses, in particular pestiviruses, such as border disease and bovine viral diarrhoea virus, and free from contamination with bacteria, fungi and/or mycoplasmas.

The general procedures for sterility or purity tests are described in Chapter 1.1.9 *Tests of biological materials for sterility and freedom from contamination*.

2.2. Method of manufacture

The method of manufacture should be documented as the Outline of Production.

2.2.1. Procedure

Vaccine batches are produced on fresh monolayers of secondary LT cells. A vial of seed virus is reconstituted with GMEM or other appropriate medium and inoculated onto an LT monolayer that has been previously washed with warm PBS, and allowed to absorb for 15 minutes at 37°C before being overlaid with additional GMEM. Cells should be harvested after 4–6 days when they exhibit 50–70% CPE for maximum viral infectivity, or earlier if CPE is extensive and they appear ready to detach. The culture is freeze–thawed three times, and the suspension is recovered and centrifuged at 600 *g* for 20 minutes. Before harvest, the culture should be examined for any evidence of nonspecific CPE, medium cloudiness or change in medium pH. A second passage may be required to produce sufficient virus for a production batch (to produce enough for 10⁶ doses, the yield from five 175 cm² flasks is required).

The procedure is repeated and the harvests (consisting of clarified supernatants) from individually numbered flasks are each mixed separately with an equal volume of sterile, chilled 5% lactalbumin hydrolysate and 10% sucrose (dissolved in double-distilled water or appropriate balanced salt solution), and transferred to individually numbered bottles for storage at –20°C. Prior to storage, 0.2 ml is removed from each bottle for sterility control. An additional 0.2 ml is removed; 2 ml pools composed of 0.2 ml samples taken from ten bottles are used for virus titration. A written record of all the procedures must be kept for all vaccine batches.

2.2.2. Requirements for substrates and media

The specification and source of all ingredients used in the manufacturing procedure should be documented and the freedom of extraneous agents: bacteria, fungi, mycoplasma and viruses should be tested. The detailed testing procedure is described in chapter 1.1.9. The use of antibiotics must meet the requirements of the licensing authority.

2.2.3. In-process control

Cells: Records of the source of the master cell stocks should be maintained. The highest and lowest passage numbers of the cells that can be used for vaccine production must be indicated in the outline of the production. In case primary lamb testis cells are used, cells should be obtained from the testis of a healthy young lamb from a scrapie-free flock of a wool sheep breed. During cultivation, cells must be observed for any evidence of CPE, and for normal morphology (predominantly fibroblastic). They can usually be passaged successfully up to ten times. When used for vaccine production, uninfected control cultures should be grown in parallel and maintained for at least one additional passage for further observation. They should be checked for the presence of noncytopathic strains of pestiviruses such as bovine viral diarrhoea or border disease viruses by immunofluorescence or immunoperoxidase or by other appropriate techniques. If possible, master cell stocks should be prepared and screened prior to vaccine production, and a stock stored in sterile DMSO (dimethyl sulphoxide) in liquid nitrogen (1–2 ml aliquots containing 20×10⁶ cells/ml).

Serum: Bovine serum used in the growth or maintenance medium must be tested free from antibody to capripoxvirus and contamination with pestivirus or any other viruses, extraneous bacteria, mycoplasma or fungi.

Medium: must be tested free from antibody to capripoxvirus and contamination with pestivirus or any other extraneous viruses, bacteria, mycoplasma or fungi.

Virus: Seed virus and final vaccine must be titrated in tissue culture tubes or microtitre plates. The minimum recommended field dose of the Kenyan and South African vaccines is log₁₀ 3.5 TCID₅₀, although the minimum protective dose is log₁₀ 2.0 TCID₅₀. Capripoxvirus is highly susceptible to inactivation by sunlight, and allowance should be made for loss of activity in the field. The recommended field dose of the Romanian sheep pox vaccine for cattle is log₁₀ 2.5 sheep infective doses (SID₅₀), and the recommended dose for cattle of the RM65-adapted strain of Romanian sheep pox vaccine is log₁₀ 3 TCID₅₀ (Coakley & Capstick, 1961). Vaccine samples must be examined for the presence of adventitious viruses including cytopathic and noncytopathic strains of pestivirus, and should be mixed with a high-titre capripoxvirus-immune serum that has previously tested negative for antibodies to pestiviruses, to prevent the vaccine virus itself from interfering with the test. The vaccine can be held at –20°C until all sterility tests

and titrations have been completed, at which time it should be freeze-dried. A further titration is carried out on five randomly chosen vials of the freeze-dried preparation to confirm the titre.

2.2.4. Final product batch tests

i) Sterility/purity

Tests of biological materials for sterility and freedom from contamination may be found in chapter 1.1.9.

ii) Safety and efficacy

The efficacy and safety studies should be demonstrated by statistically valid vaccination–challenge studies using seronegative young LSDV susceptible dairy cattle breeds. The cattle are placed in a high containment level large animal unit and serum samples are collected. Five randomly chosen vials of the freeze-dried vaccine are reconstituted in sterile PBS and pooled. Overdose studies: two cattle are inoculated with 10 times the field dose of the vaccine, the remaining vaccine is diluted with sterile PBS and two cattle are inoculated subcutaneously with the recommended field dose. The remaining two cattle are control animals. The animals are clinically examined daily and rectal temperatures are recorded. On day 21 after vaccination, the six animals are again serum sampled and challenged with a known virulent capripoxvirus strain by intradermal inoculation. (The challenge virus solution should also be tested free from extraneous viruses that can contaminate the vaccine.) The clinical response is recorded during the following 14 days. Control animals should develop the typical clinical signs of LSD, whereas there should be no local or systemic reaction in the vaccinates other than a delayed-type hypersensitivity reaction, which should disappear after 4 days. Serum samples are again collected on day 30 after vaccination. The day 21 serum samples are examined for seroconversion to selected viral diseases that could have contaminated the vaccine, and the days 0 and 30 samples are compared to confirm the absence of antibody to pestivirus. Because of the variable response in cattle to LSD challenge, generalised disease may not be seen in the control animals, although there should be a large local reaction.

The fully reconstituted vaccine is also tested in mice and guinea-pigs. Two guinea-pigs are inoculated intramuscularly with 0.5 ml into the hind leg, and two guinea-pigs and six mice are inoculated intraperitoneally with 0.5 ml and 0.1 ml, respectively. Two guinea-pigs and four mice are kept as uninoculated controls. The animals are observed for 3 weeks, humanely killed and a post-mortem examination is carried out. There should be no evidence of pathology caused by the vaccine.

iii) Batch potency

Potency tests in cattle must be undertaken for vaccine strains of capripoxvirus if the minimum immunising dose is not known. This is usually carried out by comparing the titre of a virulent challenge virus on the flanks of vaccinated and control animals. Following vaccination, the flanks of at least three animals and three controls are shaved of hair. Log₁₀ dilutions of the challenge virus are prepared in sterile PBS and six dilutions are inoculated intradermally (0.1 ml per inoculum) along the length of the flank; four replicates of each dilution are inoculated down the flank. An oedematous swelling will develop at possibly all 24 inoculation sites on the control animals, although preferably there will be little or no reaction at the four sites of the most dilute inocula. The vaccinated animals should develop an initial hypersensitivity reaction at sites of inoculation within 24 hours, which should quickly subside. Small areas of necrosis may develop at the inoculation site of the most concentrated challenge virus. The titre of the challenge virus is calculated for the vaccinated and control animals; a difference of log titre >log₁₀ 2.5 is taken as evidence of protection.

2.3. Requirements for authorisation

2.3.1. Safety requirements

i) Target and non-target animal safety

The vaccine must be safe to use in all breeds of cattle for which it is intended, including young and pregnant animals. It must also be non-transmissible and remain attenuated after further tissue culture passage.

Safety tests should be carried out on the final product of each batch as described in Section C.2.2.4.

The safety of the vaccine in non-target animals must have been demonstrated using mice and guinea-pigs as described in Section C.2.2.4. There should be no evidence of pathology caused by the vaccine.

ii) Reversion-to-virulence for attenuated/live vaccines

The selected final vaccine should not revert to virulence during further passages in target animals.

iii) Environmental consideration

Attenuated vaccine should not be able to perpetuate autonomously in a cattle population. Strains of LSDV are not a hazard to human health.

2.3.2. Efficacy requirements

i) For animal production

The efficacy of the vaccine must be demonstrated in vaccination challenge experiments under laboratory conditions. Six cattle of known susceptibility to LSD are placed in a high containment level large animal unit. Five randomly chosen vials of the freeze-dried vaccine are reconstituted in sterile PBS and pooled. Four cattle are vaccinated with recommended dose using the recommended route. The remaining two cattle are control animals. On day 21 after vaccination, the six animals are challenged with a known virulent capripoxvirus strain by intradermal inoculation. The clinical response is recorded during the following 14 days. Control animals should develop the typical clinical signs of LSD, whereas there should be no local or systemic reaction in the vaccinates other than a delayed-type hypersensitivity reaction, which should disappear after 4 days. Because of the variable response in cattle to LSD challenge, generalised disease may not be seen in the control animals, although there should be a large local reaction.

Once the potency of the particular strain being used for vaccine production has been determined in terms of minimum dose required to provide immunity, it is not necessary to repeat this on the final product of each batch, provided the titre of virus present has been ascertained.

ii) For control and eradication

Vaccination is the only effective way to control LSD outbreaks in endemic countries. Unfortunately, currently no marker vaccines allowing the differentiation of infected from vaccinated animals are available.

Immunity to virulent field virus following vaccination lasts 2 years with the Kenyan strain and 3 years with the South African vaccine, and protection against generalised infection following intradermal challenge is effectively lifelong. The duration of immunity produced by other vaccine strains should be ascertained in cattle by undertaking controlled trials in an environment in which there is no possibility of field strains of capripoxvirus interfering with the results.

2.3.3. Stability

All vaccines are initially given a shelf life of 24 months before expiry. Real-time stability studies are then conducted to confirm the appropriateness of the expiry date. Multiple batches of the vaccine should be re-titrated periodically throughout the shelf-life to determine the vaccine variability.

Properly freeze-dried preparations of LSD vaccine, particularly those that include a protectant, such as sucrose and lactalbumin hydrolysate, are stable for over 25 years when stored at –20°C and for 2–4 years when stored at 4°C. There is evidence that they are stable at higher temperatures, but no long-term controlled experiments have been reported. No preservatives other than a protectant, such as sucrose and lactalbumin hydrolysate, are required for the freeze-dried preparation.

3. Vaccines based on biotechnology

A new generation of capripox vaccines is being developed that uses the LSDV as a vector for the expression and delivery of immuno-protective proteins of other ruminant pathogens with the potential for providing dual protection (Boshra *et al.*, 2013; Wallace & Viljoen, 2005).

3.1. Vaccines available and their advantages

Currently, no recombinant capripox vaccines are commercially available.

3.2. Special requirements for biotechnology vaccines, if any

Not applicable at present.

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NB: There are OIE Reference Laboratories for Lumpy skin disease
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list:
<http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/>).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for lumpy skin disease