**Annex 12. Item 5.1 – Chapter 3.4.12 Lumpy Skin Disease**

2 MEETING OF THE WOAH BIOLOGICAL STANDARDS COMMISSION

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4

5 **CHAP T E R 3 . 4 . 1 2 .**

# 6 L U M PY SK I N D I SEASE

7 **SUMMARY**

1. ***Description of the disease:*** *Lumpy skin disease* (*LSD*) *is a poxvirus disease of cattle characterised by*
2. *fever, nodules on the skin, mucous membranes and internal organs, emaciation, enlarged lymph nodes,*
3. *oedema of the skin, and sometimes death. The disease is of economic importance as it can cause a*
4. *temporary reduction in milk production, temporary or permanent sterility in bulls, damage to hides and,*
5. *occasionally, death*. *Various strains of capripoxvirus are responsible for the disease. These are antigenically*
6. *indistinguishable from strains causing sheep pox and goat pox yet distinct at the genetic level. LSD has a*
7. *partially different geographical distribution from sheep and goat pox, suggesting that cattle strains of*
8. *capripoxvirus do not infect and transmit between sheep and goats. Transmission of LSD virus* (*LSDV*) *is*
9. *thought to be predominantly by arthropods, natural contact transmission in the absence of vectors being*
10. *inefficient. Lumpy skin disease is endemic in most ~~many~~ African and Middle Eastern countries. Between*
11. *2012 and 2022, LSD spread into south-east Europe, the Balkans, Russia and Asia as part of the Eurasian*
12. *LSD epidemic.*
13. ***Pathology:*** *the nodules are firm, and may extend to the underlying subcutis and muscle. Acute histological*
14. *key lesions consist of epidermal vacuolar changes with intracytoplasmic inclusion bodies and dermal*
15. *vasculitis. Chronic key histological lesions consist of fibrosis and necrotic sequestrae.*
16. ***Detection of the agent:*** *Laboratory confirmation of LSD is most rapid using a real-time or conventional*
17. *polymerase chain reaction* (*PCR*) *method specific for capripoxviruses in combination with a clinical history*
18. *of a generalised nodular skin disease and enlarged superficial lymph nodes in cattle. Ultrastructurally,*
19. *capripoxvirus virions are distinct from those of parapoxvirus, which causes bovine papular stomatitis and*
20. *pseudocowpox, but cannot be distinguished morphologically from orthopoxvirus virions, including cowpox*
21. *and vaccinia viruses, both of which can cause disease in cattle, although neither causes generalised*
22. *infection and both are uncommon in cattle. LSDV will grow in tissue culture of bovine, ovine or caprine origin.*
23. *In cell culture, LSDV causes a characteristic cytopathic effect and intracytoplasmic inclusion bodies that is*
24. *distinct from infection with Bovine herpesvirus 2, which causes pseudo-lumpy skin disease and produces*
25. *syncytia and intranuclear inclusion bodies in cell culture. Capripoxvirus antigens can be demonstrated in*
26. *tissue culture using immunoperoxidase or immunofluorescent staining and the virus can be neutralised using*
27. *specific antisera.*
28. *A variety of conventional and real-time PCR tests as well as isothermal amplification tests using*
29. *capripoxvirus-specific primers have been published for use on a variety of samples.*
30. ***Serological tests:*** *The virus neutralisation test* (*VNT*) *and enzyme-linked immunosorbent assays* (*ELISAs*)
31. *are widely used and have been validated. The agar gel immunodiffusion test and indirect immunofluorescent*
32. *antibody test are less specific than the VNT due to cross-reactions with antibody to other poxviruses.*
33. *Western blotting using the reaction between the P32 antigen of LSDV with test sera is both sensitive and*
34. *specific, but is difficult and expensive to carry out.*
35. ***Requirements for vaccines:*** *All strains of capripoxvirus examined so far, whether derived from cattle,*
36. *sheep or goats, are antigenically similar. Attenuated cattle strains, and strains derived from sheep and goats*
37. *have been used as live vaccines against LSDV.*

## 45 A. INTRODUCTION

1. Lumpy skin disease (LSD) was first seen in Zambia in 1929, spreading into Botswana by 1943 (Haig, 1957), and then into
2. South Africa the same year, where it affected over eight million cattle causing major economic loss. In 1957 it entered
3. Kenya, ~~at the same time as~~ associated with an outbreak of sheep pox (Weiss, 1968). In 1970 LSD spread north into the
4. Sudan, by 1974 it had spread west as far as Nigeria, and in 1977 was reported from Mauritania, Mali, Ghana and Liberia.
5. Another epizootic of LSD between 1981 and 1986 affected Tanzania, Kenya, Zimbabwe, Somalia and the Cameroon, with
6. reported mortality rates in affected cattle of 20%. The occurrence of LSD north of the Sahara desert and outside the African
7. continent was confirmed for the first time in Egypt and Israel between 1988 and 1989, and was reported again in 2006
8. (Brenner *et al.,* 2006). In the past decade, LSD occurrences have been reported in the Middle Eastern, European and
9. Asian regions (for up-to-date information, consult WOAH WAHIS interface [40](#_bookmark121)). Lumpy skin disease outbreaks tend to be
10. sporadic, depending upon animal movements, immune status, and wind and rainfall patterns affecting vector populations.
11. The principal method of transmission is thought to be mechanical by various arthropod vectors (Tuppurainen *et al*., 2015).
12. Lumpy skin disease virus (LSDV) belongs to the family *Poxviridae*, subfamily *~~Chordopoxvirinae~~ Chordopoxviridae*, and
13. genus *Capripoxvirus*. In common with other poxviruses LSDV replicates in the cytoplasm of an infected cell, forming distinct
14. perinuclear viral factories. The LSD virion is large and brick-shaped measuring 293–299nm (length) and 262–273nm
15. (width). The LSDV genome structure is also similar to other poxviruses, consisting of double-stranded linear DNA that is
16. 25% GC-rich, approximately 150,000 bp in length, and encodes around 156 open reading frames (ORFs). An inverted
17. terminal repeat sequence of 2200–2300 bp is found at each end of the linear genome. The linear ends of the genome are
18. joined with a hairpin loop. The central region of the LSDV genome contains ORFs predicted to encode proteins required
19. for virus replication and morphogenesis and exhibit a high degree of similarity with genomes of other mammalian
20. poxviruses. The ORFs in the outer regions of the LSDV genome have lower similarity and likely encode proteins involved
21. in viral virulence and host range determinants.
22. Phylogenetic analysis shows the majority of LSDV strains group into two monophyletic clusters (cluster 1.1 and 1.2)
23. (Biswas *et al.,* 2020; Van Schalkwyk *et al.,* 2021). Cluster 1.1 consists of LSDV Neethling vaccine strains that are based
24. on the LSDV/Neethling/LW-1959 vaccine strain (Kara *et al.,* 2003; Van Rooyen *et al.,* 1959; van Schalkwyk *et al.,* 2020)
25. and historic wild-type strains from South Africa. Cluster 1.2 consists of wild-type strains from southern Africa, Kenya, the
26. northern hemisphere, and the Kenyan KSGP O-240 commercial vaccine. In addition to these two clusters, there have
27. recently been recombinant LSDV strains isolated from clinical cases of LSD in the field in Russia and central Asia (Flannery
28. *et al.,* 2021; Sprygin *et al.,* 2018; 2020; Wang *et al.,* 2021). These recombinant viruses show unique patterns of accessory
29. gene alleles, consisting of sections of both wild-type and “vaccine” LSDV strains.
30. The severity of the clinical signs of LSD is highly variable and depends on a number of factors, including the strain of
31. capripoxvirus the age of the host, immunological status and breed. *Bos taurus* is generally more susceptible to clinical disease
32. than *Bos indicus;* the Asian buffalo (*Bubalus spp.*) has also been reported to be susceptible. Within *Bos taurus,* the fine-
33. skinned Channel Island breeds develop more severe disease, with lactating cows appearing to be the most at risk. However,
34. even among groups of cattle of the same breed kept together under the same conditions, there is a large variation in the
35. clinical signs presented, ranging from subclinical infection to death (Carn & Kitching, 1995). There may be failure of the virus
36. to infect the whole group, probably depending on the virulence of the virus isolate, immunological status of the host, host
37. genotype, and vector prevalence. Seroprevalence studies, experimental infections and case reports have provided indications
38. that several wildlife species (e.g. springbok, impala, giraffe, camel, banteng) are susceptible to LSDV infection (Dao *et al.,*
39. 2022; Hedger & Hamblin, 1983; Kumar *et al.,* 2023; Porco *et al.,* 2023). The scarcity of documented outbreaks in wildlife and
40. the fact that available studies remain limited in number and mostly involve only a few animals, make it difficult to determine
41. the role of wildlife in LSDV epidemiology. This topic deserves further study, especially given the current spread of LSDV in
42. new geographical areas where large numbers of naïve, potentially susceptible wild bovines and other ruminants are present.
43. The incubation period under field conditions has not been reported, but following experimental inoculation is 6–9 days until
44. the onset of fever. In the acutely infected animal, there is an initial pyrexia, which may exceed 41°C and persist for 1 week.
45. All the superficial lymph nodes become enlarged. In lactating cattle there is a marked reduction in milk yield. Lesions
46. develop over the body, particularly on the head, neck, udder, scrotum, vulva and perineum between 7 and 19 days after
47. virus inoculation (Coetzer, 2004). The characteristic integumentary lesions are multiple, well circumscribed to coalescing,
48. 0.5–5 cm in diameter, firm, flat-topped papules and nodules. The nodules involve the dermis and epidermis, and may
49. https://[www.woah.org/en/what-we-do/animal-health-and-welfare/disease-data-collection/](http://www.woah.org/en/what-we-do/animal-health-and-welfare/disease-data-collection/)
50. extend to the underlying subcutis and occasionally to the adjacent striated muscle. These nodules have a creamy grey to
51. white colour on cut section, which may initially exude serum, but over the ensuing 2 weeks a cone-shaped central core or
52. sequestrum of necrotic material/necrotic plug (“sit-fast”) may appear within the nodule. The acute histological lesions
53. consist of epidermal vacuolar changes with intracytoplasmic inclusion bodies and dermal vasculitis. The inclusion bodies
54. are numerous, intracytoplasmic, eosinophilic, homogenous to occasionally granular and they may occur in endothelial
55. cells, fibroblasts, macrophages, pericytes, and keratinocytes. The dermal lesions include vasculitis with fibrinoid necrosis,
56. oedema, thrombosis, lymphangitis, dermal-epidermal separation, and mixed inflammatory infiltrate. The chronic lesions
57. are characterised by an infarcted tissue with a sequestered necrotic core, often rimmed by granulation tissue gradually
58. replaced by mature fibrosis. At the appearance of the nodules, the discharge from the eyes and nose becomes
59. mucopurulent, and keratitis may develop. Nodules may also develop in the mucous membranes of the mouth and
60. alimentary tract, particularly the abomasum and in the trachea and the lungs, resulting in primary and secondary
61. pneumonia. The nodules on the mucous membranes of the eyes, nose, mouth, rectum, udder and genitalia quickly
62. ulcerate, and by then all secretions, ocular and nasal discharge and saliva contain LSD virus (LSDV). The limbs may be
63. oedematous and the animal is reluctant to move. Pregnant cattle may abort, and there is a report of intrauterine
64. transmission (Rouby & Aboulsoudb, 2016). Bulls may become permanently or temporarily infertile and the virus can be
65. excreted in the semen for prolonged periods (Irons *et al*., 2005). Recovery from severe infection is slow; the animal is
66. emaciated, may have pneumonia and mastitis, and the necrotic plugs of skin, which may have been subject to fly strike,
67. are shed leaving deep holes in the hide (Prozesky & Barnard, 1982).
68. The main differential diagnosis is pseudo-LSD caused by bovine herpesvirus 2 (BoHV-2). This is usually a milder clinical
69. condition, characterised by superficial nodules, resembling only the early stage of LSD. Intra-nuclear inclusion bodies and
70. viral syncytia are histopathological characteristics of BoHV-2 infection not seen in LSD. Other differential diagnoses (for
71. integumentary lesions) include: dermatophilosis, dermatophytosis, bovine farcy, photosensitisation, actinomycosis,
72. actinobacillosis, urticaria, insect bites, besnoitiosis, nocardiosis, demodicosis, onchocerciasis, pseudo-cowpox, and
73. cowpox. Differential diagnoses for mucosal lesions include: foot and mouth disease, bluetongue, mucosal disease,
74. malignant catarrhal fever, infectious bovine rhinotracheitis, and bovine papular stomatitis.
75. LSDV is not transmissible to humans. However, all laboratory manipulations must be performed at an appropriate
76. containment level determined using biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing*
77. *biological risk in the veterinary laboratory and animal facilities*)*.*

## 122 B. DIAGNOSTIC TECHNIQUES

123 ***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 |
| **Detection of the agent** | | | | | | |
| **Virus isolation** | + | ++ | + | +++ | + | – |
| **PCR** | ++ | +++ | ++ | +++ | + | – |
| **TEM** | – | – | – | + | – | – |
| **Detection of immune response** | | | | | | |
| **VNT** | ++ | ++ | ++ | ++ | ++ | ++ |
| **IFAT** | + | + | + | + | + | + |
| **ELISA** | ++ | ++ | ++ | ++ | ++ | ++ |

124 Key: +++ = recommended for this purpose; ++ recommended but has limitations;

125 + = suitable in very limited circumstances; – = not appropriate for this purpose.

1. PCR = polymerase chain reaction; TEM = Transmission electron microscopy; VNT = virus neutralisation test;
2. IFAT = indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay.

#### 1. Detection of the agent

##### 1.1. Specimen collection, submission and preparation

1. Material for virus isolation and antigen detection should be collected as biopsies or from skin nodules at post-mortem
2. examination. Samples for virus isolation should preferably be collected within the first week of the occurrence of
3. clinical signs, before the development of neutralising antibodies (Davies, 1991; Davies *et al*., 1971), however virus
4. can be isolated from skin nodules for at least 3–4 weeks thereafter. Samples for genome detection using conventional
5. or real-time polymerase chain reaction (PCR) may be collected when neutralising antibody is present. Following the
6. first appearance of the skin lesions, the virus can be isolated for up to 35 days and viral nucleic acid can be
7. demonstrated via PCR for up to 3 months (Tuppurainen *et al*., 2005; Weiss, 1968). Buffy coat from blood collected
8. into heparin or EDTA (ethylene diamine tetra-acetic acid) during the viraemic stage of LSD (before generalisation of
9. lesions or within 4 days of generalisation), can also be used for virus isolation. Samples for histology should include
10. the lesion and tissue from the surrounding (non-lesion) area, be a maximum size of 2 cm3, and be placed immediately
11. following collection into ten times the sample volume of 10% neutral buffered formal saline.
12. Tissues in formalin have no special transportation requirements in regard to biorisks. Blood samples with
13. anticoagulant for virus isolation from the buffy coat should be placed immediately on ice after gentle mixing and
14. processed as soon as possible. In practice, the samples may be kept at 4°C for up to 2 days prior to processing, but
15. should not be frozen or kept at ambient temperatures. Tissues for virus isolation and antigen detection should be kept
16. at 4°C, on ice or at –20°C. If it is necessary to transport samples over long distances without refrigeration, the medium
17. should contain 10% glycerol; the samples should be of sufficient size (e.g. 1 g in 10 ml) that the transport medium
18. does not penetrate the central part of the biopsy, which should be used for virus isolation.
19. ~~Samples for histology should include the lesion and tissue from the surrounding (non-lesion) area, be a maximum~~
20. ~~size of 2 cm~~3~~, and be placed immediately following collection into ten times the sample volume of 10% neutral buffered~~
21. ~~formaldehyde. Tissues in formalin have no special transportation requirements in regard to biorisks.~~ Material for
22. histology should be prepared using standard techniques and stained with haematoxylin and eosin (H&E) (Burdin,
23. 1959). Lesion material for virus isolation and antigen detection is minced using a sterile scalpel blade and forceps
24. and then macerated in a sterile steel ball-bearing mixer mill, or ground with a pestle in a sterile mortar with sterile
25. sand and an equal volume of sterile phosphate buffered saline (PBS) or serum-free modified Eagle’s medium
26. containing sodium penicillin (1000 international units [IU]/ml), streptomycin sulphate (1 mg/ml), mycostatin
27. (100 IU/ml) or fungizone (amphotericin, 2.5 µg/ml) and neomycin (200 IU/ml). The suspension is freeze–thawed three
28. times and then partially clarified using a bench centrifuge at 600 ***g*** for 10 minutes. In cases where bacterial
29. contamination of the sample is expected (such as when virus is isolated from skin samples), the supernatant can be
30. filtered through a 0.45 µm pore size filter after the centrifugation step. Buffy coats may be prepared from unclotted
31. blood using centrifugation at 600 ***g*** for 15 minutes, and the buffy coat carefully removed into 5 ml of cold double-
32. distilled water using a sterile Pasteur pipette. After 30 seconds, 5 ml of cold double-strength growth medium is added
33. and mixed. The mixture is centrifuged at 600 ***g*** for 15 minutes, the supernatant is discarded and the cell pellet is
34. suspended in 5 ml growth medium, such as Glasgow’s modified Eagle’s medium (GMEM). After centrifugation at
35. 600 ***g*** for a further 15 minutes, the resulting pellet is suspended in 5 ml of fresh GMEM. Alternatively, the buffy coat
36. may be separated from a heparinised sample by using a Ficoll gradient.

##### 1.2. Virus isolation on cell culture

1. LSDV will grow in tissue culture of bovine, ovine or caprine origin. MDBK (Madin–Darby bovine kidney) cells are often
2. used, as they support good growth of the virus and are well characterised (Fay *et al.,* 2020). Primary cells, such as
3. lamb testis (LT) cells also support viral growth, but care needs to be taken to ensure they are not contaminated with
4. viruses such as bovine viral diarrhoea virus. One ml of clarified supernatant or buffy coat is inoculated onto a confluent
5. monolayer in a 25 cm2 culture flask at 37°C and allowed to adsorb for 1 hour. The culture is then washed with warm
6. PBS and covered with 10 ml of a suitable medium, such as GMEM, containing antibiotics and 2% fetal calf serum. If
7. available, tissue culture tubes containing appropriate cells and a flying cover-slip, or tissue culture microscope slides,
8. are also infected.
9. The flasks/tissue culture tubes are examined daily for 7–14 days for evidence of cytopathic effects (CPE). Infected
10. cells develop a characteristic CPE consisting of retraction of the cell membrane from surrounding cells, and eventually
11. rounding of cells and margination of the nuclear chromatin. At first only small areas of CPE can be seen, sometimes
12. as soon as 2 days after infection; over the following 4–6 days these expand to involve the whole cell monolayer ~~sheet~~.
13. If no CPE is apparent by day 14, the culture should be freeze–thawed three times, and clarified supernatant inoculated
14. on to a fresh cell monolayer. At the first sign of CPE in the flasks, or earlier if a number of infected cover-slips are
15. being used, a cover-slip should be removed, fixed in acetone and stained using H&E. Eosinophilic intracytoplasmic
16. inclusion bodies, which are variable in size but up to half the size of the nucleus and surrounded by a clear halo, are
17. diagnostic for poxvirus infection. PCR may be used as an alternative to H&E for confirmation of the diagnosis. The
18. CPE can be prevented or delayed by adding specific anti-LSDV serum to the medium. In contrast, the herpesvirus
19. that causes pseudo-LSD produces a Cowdry type A intranuclear inclusion body. It also forms syncytia.
20. An ovine testis cell line (OA3.T~~s~~) has been evaluated for the propagation of capripoxvirus isolates (Babiuk *et al.*,
21. 2007), however this cell line has been found to be contaminated with pestivirus and should be used with caution.

##### 1.3. Polymerase chain reaction (PCR)

1. The conventional gel-based PCR method described below is a simple, fast and sensitive method for the detection of
2. capripoxvirus genome in EDTA blood, semen or tissue culture samples (Tuppurainen *et al*., 2005).
3. 1.3.1. Test procedure
4. The extraction method described below can be replaced using commercially available DNA extraction
5. kits.
6. i) Freeze and thaw 200 µl of blood in EDTA, semen or tissue culture supernatant and suspend in
7. 100 µl of lysis buffer containing 5 M guanidine thiocyanate, 50 mM potassium chloride, 10 mM
8. Tris/HCl (pH 8); and 0.5 ml Tween 20.
9. ii) Cut skin and other tissue samples into fine pieces using a sterile scalpel blade and forceps.
10. Grind with a pestle in a mortar. Suspend the tissue samples in 800 µl of the above mentioned
11. lysis buffer.
12. iii) Add 2 µl of proteinase K (20 mg/ml) to blood samples and 10 µl of proteinase K (20 mg/ml) to
13. tissue samples. Incubate at 56°C for 2 hours or overnight, followed by heating at 100°C for
14. 10 minutes. Add phenol:chloroform:isoamylalcohol (25:24:1 [v/v]) to the samples in a 1:1 ratio.
15. Vortex and incubate at room temperature for 10 minutes. Centrifuge the samples at 16,060 ***g***
16. for 15 minutes at 4°C. Carefully collect the upper, aqueous phase (up to 200 µl) and transfer
17. into a clean 2.0 ml tube. Add two volumes of ice cold ethanol (100%) and 1/10 volume of 3 M
18. sodium acetate (pH 5.3). Place the samples at –20°C for 1 hour. Centrifuge again at 16,060 ***g***
19. for 15 minutes at 4°C and discard the supernatant. Wash the pellets with ice cold 70% ethanol
20. (100 µl) and centrifuge at 16,060 ***g*** for 1 minute at 4°C. Discard the supernatant and dry the
21. pellets thoroughly. Suspend the pellets in 30 µl of nuclease-free water and store immediately at
22. –20°C (Tuppurainen *et al*., 2005). Alternatively a column-based extraction kit may be used.
23. iv) The primers for this PCR assay were developed from the gene encoding the viral attachment
24. protein. The size of the expected amplicon is 192 bp (Ireland & Binepal, 1998). The primers
25. have the following gene sequences:
26. Forward primer 5’-TCC-GAG-CTC-TTT-CCT-GAT-TTT-TCT-TAC-TAT-3’
27. Reverse primer 5’-TAT-GGT-ACC-TAA-ATT-ATA-TAC-GTA-AAT-AAC-3’.
28. v) DNA amplification is carried out in a final volume of 50 µl containing: 5 µl of 10 × PCR buffer,
29. 1.5 µl of MgCl2 (50 mM), 1 µl of dNTP (10 mM), 1 µl of forward primer, 1 µl of reverse primer,
30. 1 µl of DNA template (~10 ng), 0.5 µl of *Taq* DNA polymerase and 39 µl of nuclease-free water.
31. The volume of DNA template required may vary and the volume of nuclease-free water must
32. be adjusted to the final volume of 50 µl.
33. vi) Run the samples in a thermal cycler as follows: 2 minutes at 95°C; then 45 seconds at 95°C,
34. 50 seconds at 50°C and 1 minute at 72°C (34 cycles); 2 minutes at 72°C and hold at 4°C until
35. analysis.
36. vii) Mix 10 µl of each sample with loading dye and load onto a 1.5% agarose gel in TAE buffer
37. (Tris/acetate buffer containing EDTA). Load a parallel lane with a 100 bp DNA-marker ladder.
38. Electrophoretically separate the products using approximately 8–10 V/cm for 40–60 minutes
39. and visualise with a suitable DNA stain and transilluminator.
40. Quantitative real-time PCR methods have been described that are reported to be faster and have
41. higher sensitivity than conventional PCRs (Balinsky *et al*., 2008; Bowden *et al*., 2008). A real-time
42. PCR method that differentiates between LSDV, sheep pox virus and goat pox virus has been
43. published (Lamien *et al.,* 2011).
44. Quantitative real-time PCR assays have been designed to differentiate between Neethling-based
45. LSDV strains, which are often used for vaccination, and wild-type LSDV strains from cluster 1.2
46. (Agianniotaki *et al.,* 2017; Pestova *et al.,* 2018; Vidanovic *et al.,* 2016). These “DIVA” assays (DIVA:
47. differentiation of infected from vaccinated animals) enable, for example, differentiation of “Neethling
48. response” caused by vaccination with a LSDV Neethling vaccine strain from disease caused by
49. infection with a cluster 1.2 wild-type virus. However these DIVA PCR assays cannot distinguish
50. between a LSDV Neethling vaccine strain and the novel recombinant LSDV strains ~~recently~~ isolated
51. from disease outbreaks in Asia (~~Byadovskaya~~ *~~et al.,~~* ~~2021;~~ Flannery *et al.,* 2021). These DIVA assays
52. are also not capable of discriminating between LSDV Neethling vaccine strains and recently
53. characterised (historic) wild-type viruses from South Africa belonging within cluster 1.1 (Van
54. Schalkwyk *et al.,* 2020; 2021). Consequently, in regions where recombinant strains (currently Asia
55. and possibly elsewhere) or wild-type cluster 1.1 strains are circulating (currently South Africa and
56. possibly elsewhere), these DIVA assays are not suitable for distinguishing vaccine and wild-type
57. virus. Thus, in order to overcome these constraints, whole genome sequencing is recommended.

##### 1.4. Transmission electron microscopy

1. The characteristic poxvirus virion can be visualised using a negative staining preparation technique followed by
2. examination with an electron microscope. There are many different negative staining protocols, an example of which
3. is given below.
4. 1.4.1. Test procedure
5. Before centrifugation, material from the original biopsy suspension is prepared for examination under
6. the transmission electron microscope by floating a 400-mesh hexagonal electron microscope grid,
7. with pioloform-carbon substrate activated by glow discharge in pentylamine vapour, onto a drop of
8. the suspension placed on parafilm or a wax plate. After 1 minute, the grid is transferred to a drop of
9. Tris/EDTA buffer, pH 7.8, for 20 seconds and then to a drop of 1% phosphotungstic acid, pH 7.2, for
10. 10 seconds. The grid is drained using filter paper, air-dried and placed in the electron microscope.
11. The capripox virion is brick shaped, covered in short tubular elements and measures approximately
12. 290 × 270 nm. A host-cell-derived membrane may surround some of the virions, and as many as
13. possible should be examined to confirm their appearance (Kitching & Smale, 1986).
14. The ~~capripox~~ virions of capripoxvirus are indistinguishable from those of orthopoxvirus, but, apart
15. from vaccinia virus and cowpox virus, which are both uncommon in cattle and do not cause
16. generalised infection, no other orthopoxvirus is known to cause lesions in cattle. However, vaccinia
17. virus may cause generalised infection in young immunocompromised calves. In contrast,
18. orthopoxviruses are a common cause of skin disease in domestic buffalo (*Bubalus bubalis*) causing
19. buffalo pox, a disease that usually manifests as pock lesions on the teats, but may cause skin lesions
20. at other sites, such as the perineum, the medial aspects of the thighs and the head. Orthopoxviruses
21. that cause buffalo pox cannot be readily distinguished from capripoxvirus by electron microscopy.
22. The virions of parapoxvirus ~~virions~~ that cause bovine papular stomatitis and pseudocowpox are
23. smaller, oval in shape and each is covered in a single continuous tubular element that appears as
24. striations over the virion. Capripoxvirus virions are also distinct from the herpesvirus that causes
25. pseudo-LSD (also known as “Allerton” or bovine herpes mammillitis).

##### 1.5. Fluorescent antibody tests

1. Capripoxvirus antigen can be identified on infected cover-slips or tissue culture slides using fluorescent antibody
2. tests. Cover-slips or slides should be washed and air-dried and fixed in cold acetone for 10 minutes. The indirect test
3. using immune cattle sera is subject to high background colour and nonspecific reactions. However, a direct conjugate
4. can be prepared from sera from convalescent cattle (or from sheep or goats convalescing from capripox) or from
5. rabbits hyperimmunised with purified capripoxvirus. Uninfected tissue culture should be included as a negative control
6. as cross-reactions can cause problems due to antibodies to cellular components (pre-absorption of these from the
7. immune serum helps solve this issue).

##### 1.6. Immunohistochemistry

1. Immunohistochemistry using F80G5 monoclonal antibody specific for capripoxvirus ORF 057 has been described for
2. detection of LSDV antigen in the skin of experimentally infected cattle (Babiuk *et al.,* 2008).

##### 1.7. Isothermal genome amplification

1. Molecular tests using loop-mediated isothermal amplification to detect capripoxvirus genomes are reported to provide
2. sensitivity and specificity similar to real-time PCR with a simpler method and lower cost (Das *et al.,* 2012; Murray *et*
3. *al.,* 2013). Field validation of the Das *et al.* method was reported by Omoga *et al.* (2016).

#### 2. Serological tests

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

##### 2.1. Virus neutralisation

1. A test serum can either be titrated against a constant titre of capripoxvirus (100 TCID50 [50% tissue culture infective
2. dose]) or a standard virus strain can be titrated against a constant dilution of test serum in order to calculate a
3. neutralisation index. Because of the variable sensitivity of tissue culture to capripoxvirus, and the consequent difficulty
4. of ensuring the accurate and repeatable seeding of 100 TCID50/well, the neutralisation index is the preferred method
5. in most laboratories, although it does require a larger volume of test sera. The test is described using 96-well flat-
6. bottomed tissue-culture grade microtitre plates, but it can be performed equally well in tissue culture tubes with the
7. appropriate changes to the volumes used, although it is more difficult to read an end-point in tubes.
8. 2.1.1. Test procedure
9. i) Test sera, including a negative and a positive control, are diluted 1/5 in Eagle’s/HEPES (N-2-
10. hydroxyethylpiperazine, N-2-ethanesulphonic acid) buffer and inactivated at 56°C for
11. 30 minutes.
12. ii) Next, 50 µl of the first inactivated serum is added to columns 1 and 2, rows A to H of the
13. microtitre plate. The second serum is placed in columns 3 and 4, the third in columns 5 and 6,
14. the positive control serum is placed in columns 7 and 8, the negative control serum is placed in
15. columns 9 and 10, and 50 µl of Eagle’s/HEPES buffer (without serum) is placed in columns 11
16. and 12, and to all wells in row H.
17. iii) A reference strain of capripoxvirus, usually a vaccine strain known to grow well in tissue culture,
18. with a titre of over log10 6 TCID50 per ml is diluted in Eagle’s/HEPES in bijoux bottles to give a
19. 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,
20. 2.7, 2.2, 1.7, 1.2, 0.7, 0.2 TCID50 per 50 µl).
21. iv) Starting with row G and the most diluted virus preparation, 50 µl of virus is added to each well
22. in that row. This is repeated with each virus dilution, the highest titre virus dilution being placed
23. in row A.
24. v) The plates are covered and incubated for 1 hour at 37°C.
25. vi) An appropriate cell suspension (such as MDBK cells) is prepared from pregrown monolayers
26. as a suspension of 105 cells/ml in Eagle’s medium containing antibiotics and 2% fetal calf serum.
27. Following incubation of the microtitre plates, 100 µl of cell suspension is added to all the wells,
28. except wells H11 and H12, which serve as control wells for the medium. The remaining wells of
29. row H are cell and serum controls.
30. vii) The microtitre plates are covered and incubated at 37°C for 9 days.
31. viii) Using an inverted microscope, the monolayers are examined daily from day 4 for evidence of
32. CPE. There should be no CPE in the cells of row H. Using the 0240 KSGP vaccine strain of
33. capripoxvirus, by way of example, the final reading is taken on day 9, and the titre of virus in
34. each duplicate titration is calculated using the Kärber method. If left longer, there is invariably a
35. ‘breakthrough’ of virus in which virus that was at first neutralised appears to disassociate from
36. the antibody.
37. ix) *Interpretation of the results:* The neutralisation index is the log titre difference between the titre
38. of the virus in the negative serum and in the test serum. An index of ≥1.5 is positive. The test
39. can be made more sensitive if serum from the same animal is examined before and after
40. infection. Because the immunity to capripoxviruses is predominantly cell mediated, a negative
41. result, particularly following vaccination, after which the antibody response may be low, does
42. not imply that the animal from which the serum was taken is not protected.
43. x) Antibodies to capripoxvirus can be detected from 1 to 2 days after the onset of clinical signs.
44. These remain detectable for about 7 months.

##### 2.2. Enzyme-linked immunosorbent assay

1. Enzyme-linked immunosorbent assays (ELISAs) for the detection of capripoxviral antibodies are widely used and are
2. available in commercial kit form (Milovanovic *et al.,* 2019; Samojlovic *et al.,* 2019).

##### 2.3. Indirect fluorescent antibody test

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

##### 2.4. Western blot analysis

1. Western blotting of test sera against capripoxvirus-infected cell lysate provides a sensitive and specific system for
2. the detection of antibody to capripoxvirus structural proteins, although the test is expensive and difficult to carry out.
3. Capripoxvirus-infected LT cells should be harvested when 90% CPE is observed, freeze–thawed three times, and
4. the cellular debris pelleted using centrifugation. The supernatant should be decanted, and the proteins should be
5. separated using SDS/PAGE (sodium dodecyl sulphate/polyacrylamide gel electrophoresis). A vertical discontinuous
6. gel system, using a stacking gel made up of acrylamide (5%) in Tris (125 mM), pH 6.8, and SDS (0.1%), and a
7. resolving gel made up of acrylamide (10–12.5%) in Tris (560 mM), pH 8.7, and SDS (0.1%), is recommended for use
8. with a glycine running buffer containing Tris (250 mM), glycine (2 M), and SDS (0.1%). Samples of supernatant should
9. be prepared by boiling for 5 minutes with an appropriate lysis buffer prior to loading. Alternatively, purified virus or
10. recombinant antigens may replace tissue-culture-derived antigen.
11. Molecular weight markers should be run concurrently with the protein samples. The separated proteins in the
12. SDS/PAGE gel should be transferred electrophoretically to a nitrocellulose membrane (NCM). After transfer, the NCM
13. is rinsed thoroughly in PBS and blocked in 3% bovine serum albumin (BSA) in PBS, or 5% skimmed milk powder in
14. PBS, on a rotating shaker at 4C overnight. The NCM can then be separated into strips by employing a commercial
15. apparatus to allow the concurrent testing of multiple serum samples, or may be cut into strips and each strip incubated
16. separately thereafter. The NCM is washed thoroughly with five changes of PBS for 5 minutes on a rotating shaker,
17. and then incubated at room temperature on the shaker for 1.5 hours, with the appropriate serum at a dilution of 1/50
18. in blocking buffer (3% BSA and 0.05% Tween 20 in PBS; or 5% milk powder and 0.05% Tween 20 in PBS). The
19. membrane is again thoroughly washed and incubated (in blocking buffer) with anti-species immunoglobulin
20. horseradish-peroxidase-conjugated immunoglobulins at a dilution determined using titration. After further incubation
21. at room temperature for 1.5 hours, the membrane is washed and a solution of diaminobenzidine tetrahydrochloride
22. (10 mg in 50 ml of 50 ~~mM~~ mm Tris/HCl, pH 7.5, and 20 µl of 30% [v/v] hydrogen peroxide) is added. Incubation is
23. then undertaken for approximately 3–7 minutes at room temperature on a shaker with constant observation, and the
24. reaction is stopped by washing the NCM in PBS before excessive background colour is seen. A positive and negative
25. control serum should be used on each occasion.
26. Positive test samples and the positive control will produce a pattern consistent with reaction to proteins of molecular
27. weights 67, 32, 26, 19 and 17 kDa – the major structural proteins of capripoxvirus – whereas negative serum samples
28. will not react with all these proteins. Hyperimmune serum prepared against parapoxvirus (bovine papular stomatitis
29. or pseudocowpox virus) will react with some of the capripoxvirus proteins, but not the 32 kDa protein that is specific
30. for capripoxvirus.

## 377 C. REQUIREMENTS FOR VACCINES

#### 1. Background: rationale and intended use of the product

1. Live attenuated strains of capripoxvirus have been used as vaccines specifically for the control of LSD (Brenner *et al*.,
2. 2006; Capstick & Coakley, 1961; Carn, 1993). Capripoxviruses are cross-reactive within the genus. Consequently, it is
3. possible to protect cattle against LSD using strains of capripoxvirus derived from sheep or goats (Coakley & Capstick,
4. 1961). However, it is recommended to carry out controlled trials, using the most susceptible breeds, prior to introducing a
5. ~~vaccine strain not usually used in cattle. The duration of protection provided by LSD vaccination is unknown.~~
6. Capripoxvirus vaccine strains can produce a large local reaction at the site of inoculation in *Bos taurus* breeds (Davies,
7. 1991), which some stock owners find unacceptable. This has discouraged the use of vaccine, even though the
8. consequences of an outbreak of LSD are invariably more severe. Risk–benefit of vaccination should be assessed following
9. ~~stakeholder discussion.~~
10. Vaccines are a key tool to control LSD. Different types of LSD vaccines have been developed and several are commercially
11. available (Tuppurainen *et al.,* 2021).
12. Live attenuated vaccines (LAV) based on the Neethling LSDV strain (homologous LAV vaccines) have been shown to offer
13. high levels of protection against LSD under experimental conditions (Haegeman *et al.,* 2021) and have been used
14. successfully to control the disease in the field, through systematic vaccination of the entire country’s cattle population for
15. a number of consecutive years (Klement *et al.,* 2020). Homologous vaccines may induce fever, produce a local reaction
16. at the site of inoculation, cause a temporary reduction in milk production and on rare occasions induce a ‘Neethling’
17. response (Ben-Gera *et al.,* 2015; Davies, 1991; Haegeman *et al.,* 2021). Such adverse effects, however, usually resolve
18. within a few days and are largely outweighed by the overall benefits of vaccination with homologous vaccines. The duration
19. of immunity induced by good quality live attenuated LSDV vaccines was shown to be at least 18 months (Haegeman *et*
20. *al.,* 2023).
21. As capripox viruses provide cross-reactive protection within the genus, heterologous LAVs comprising sheep pox virus or
22. goat pox virus strains have also been tested and used to protect cattle against LSD. Sheep pox virus-based heterologous
23. vaccines usually contain higher doses of virus than when administered to sheep. Although safe, their effectiveness in
24. protecting cattle against LSD is inferior compared to homologous vaccines (Ben-Gera *et al*., 2015; Zhugunissov *et al*.,
25. 2020). Heterologous vaccines containing goat pox virus strains for use in cattle against LSD have been developed more
26. recently. One such vaccine based on the Gorgan strain provided protection under experimental conditions comparable to
27. homologous vaccines (Gari *et al*., 2015). On the other hand, a goat pox vaccine based on an attenuated Uttarkashi goat
28. pox virus strain performed suboptimally under field conditions in India (Naveem *et al*., 2023), indicating that further research
29. is warranted before asserting that all goat pox virus-based vaccines induce protection equal to homologous vaccines in
30. cattle against LSD.
31. In addition, homologous inactivated vaccines against LSD have been developed and tested (Haegeman *et al*., 2023; Hamdi
32. *et al*., 2020; Wolff *et al*., 2022). These vaccines are reported to be safe and efficacious. They however require a booster
33. vaccination one month after primo-vaccination and then every 6 months thereafter, based on the fact that the duration of
34. immunity is shorter than 1 year (Haegeman *et al*., 2023).
35. None of the commercial vaccines currently available has practical DIVA capacity. This problem may be resolved in the
36. future by introducing new types of vaccines (e.g. vector-vaccines, subunit vaccines, mRNA vaccines) that are at various
37. stages of development and evaluation.

#### 2. Outline of production of LSD vaccines ~~and minimum requirements for conventional~~

1. **~~vaccines~~**
2. ~~General requirements set for the facilities used for the production of vaccines and for the documentation and record keeping~~
3. ~~throughout the whole manufacturing process are described in Chapter 1.1.8~~ *~~Principles of veterinary vaccine production~~*~~.~~
4. ~~The documentation should include standard operating procedures (SOP) for the method of manufacture and each step for~~
5. ~~the testing of cells and reagents used in the process, each batch and the final product.~~
6. The production of vaccines, including LSD vaccines, starts within research and development (R&D) facilities where vaccine
7. candidates are produced and tested in preclinical studies to demonstrate the quality, safety and efficacy of the product.
8. Minimum requirements for different production stages of veterinary vaccines are available in different chapters of the
9. *Terrestrial Manual.* These are intended to be used in combination with country-specific regulatory requirements for vaccine
10. production and release. Here we outline the most important requirements for the production of live and inactivated LSD
11. vaccines. Full requirements are available in Chapter 1.1.8 *Principles of veterinary vaccine production,* Chapter 2.3.3
12. *Minimum requirements for the organisation and management of a vaccine manufacturing facility* and Chapter 2.3.4
13. *Minimum requirements for the production and quality control of vaccine,* and other regulatory documentation*.*

##### 2.1. Quality assurance

1. Facilities for manufacturing LSD vaccines should operate in line with the concepts of good laboratory practice (GLP)
2. and good manufacturing practice (GMP) to produce high quality products. Quality risk management and quality
3. control with adequate documentation management, as an integral part of the production process, have to be in place.
4. In case some activities of the production process are outsourced, those should also be appropriately defined,
5. recorded and controlled.
6. The vaccine production process (Outline of Production) should be documented in a series of standard operating
7. procedures (SOPs), or other documents describing the manufacturing of each batch and the final product (including
8. starting materials to be used, manufacturing steps, in-process controls and controls on the final product). Detailed
9. requirements for documentation management in the process of vaccine production are available in Chapter 2.3.3.
10. A completed Outline of Production is to be enclosed in a vaccine candidate dossier and used for the evaluation of
11. the production process and product by regulatory bodies.

##### 2.2. Process validation

1. The dossier with the enclosed Outline of Production for the vaccine candidate has to be submitted for regulatory
2. approval, so it can be assessed and authorised by the competent authority to ensure compliance with local regulatory
3. requirements. Among others, data on quality, safety, and efficacy will be assessed. The procedures necessary to
4. obtain these data are described in the subsequent sections.
5. National regulatory authorities might also require official control authority re-testing (check testing) of final products
6. and batches in government laboratories or an independent batch quality control by a third party.
7. **3. Requirements for LSD vaccine candidates and batch production**

##### 3.1. Requirements for starting materials

1. Live attenuated vaccines (LAV) and inactivated vaccines (IV) for LSD are produced using the system of limited and
2. controlled passages of master seed and working seed virus and cell banks with a specified maximum. This approach
3. aims to prevent possible and unwanted drift of properties of seed virus and cells that might arise from repeated
4. passaging.
5. 3.1.1. Characteristics of the seed virus
6. ~~Each seed strain of capripoxvirus used for vaccine production must be accompanied by records~~
7. ~~clearly and accurately describing its origin, isolation and tissue culture or animal passage history.~~
8. ~~Preferably, the species and strain of capripoxvirus are characterised using PCR or DNA sequencing~~
9. ~~techniques.~~
10. ~~A quantity of master seed vaccine virus should be prepared, frozen or desiccated and stored at low~~
11. ~~temperatures such as –80°C and used to produce a consistent working seed for regular vaccine~~
12. ~~production.~~
13. ~~Each master seed strain must be non-transmissible, remain attenuated after further tissue culture~~
14. ~~passage, and provide complete protection against challenge with virulent field strains for a minimum~~
15. ~~of 1 year. It must produce a minimal clinical reaction in cattle when given via the recommended route.~~
16. ~~The necessary safety and potency tests are described in Section C.2.2.4~~ *~~Final product batch tests~~*~~.~~
17. ~~2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)~~
18. ~~Each master seed must be tested to ensure its identity and shown to be free from adventitious~~
19. ~~viruses, in particular pestiviruses, such as border disease and bovine viral diarrhoea virus, and free~~
20. ~~from contamination with bacteria, fungi or mycoplasmas.~~
21. ~~The general procedures for sterility or purity tests are described in Chapter 1.1.9~~ *~~Tests for sterility~~*
22. *~~and freedom from contamination of biological materials intended for veterinary use~~*~~.~~
23. Master seed virus is a quantity of virus of uniform composition derived from an original isolate,
24. passaged for a documented number of times and distributed into containers at one time and stored
25. adequately to ensure stability (via freezing or lyophilisation). Selection of master seed viruses (MSVs)
26. should ideally be based on their ease of growth in cell culture, virus yield, and in accordance with the
27. regional epidemiological importance. Also, measures to minimise transmissible spongiform
28. encephalopathies (TSE) contamination should be taken into account (see Section C.3.5.1 *Purity*
29. *tests*).
30. For each seed strain selected for LSD vaccine production, the following information should be
31. provided:
32. - Historical record: geographical origin, animal species from which the virus was recovered,
33. isolation procedure, tissue culture or animal passage history
34. - Identity: species and strain identification using DNA sequencing
35. - Purity: the absence of bacteria, fungi, mycoplasma, and other viruses (see Chapter 1.1.9 *Tests*
36. *for sterility and freedom from contamination of biological materials intended for veterinary use*)
37. - Safety (overdose, one/repeated dose tests, and reversion to virulence tests) (see Section C.3.3
38. *Vaccine safety*)
39. - Efficacy data, linked to a specified (protective) dose (see Section C.3.4 *Vaccine efficacy*)
40. - Stability
41. Each master seed strain selected for production of live attenuated LSD vaccines must remain
42. attenuated after further passage in animals (see Section C.3.3. *Vaccine safety*), produce minimal
43. clinical reaction when given via the recommended route, provide complete protection against
44. challenge with virulent field strains, and is ideally not transmissible.
45. A quantity of master seed virus should be prepared and stored to be further used for the preparation
46. of working seeds and production seeds. Working seed viruses may be expanded in one or more (but,
47. limited) cell culture passages from the master seed stock and used to produce vaccine batches. This
48. approach and limitation of seed virus passaging will assist in maintaining uniformity and consistency
49. in production.
50. 3.1.2. Master cell stocks
51. The production process of LSD vaccines ideally employs an established master cell stock (MCS)
52. system with defined lowest and highest cell passage to be used to grow the vaccine virus. Primary
53. cells derived from normal tissues can be used in the production process, but the use of primary cells
54. has an inherently higher risk of introducing extraneous agents compared with the use of established
55. (well characterised) cell lines and should be avoided where alternative methods of producing effective
56. vaccines exist. For each MCS, manufacturers should demonstrate:
57. - MCS identity
58. - genetic stability by subculturing from the lowest to the highest passage used for production
59. - stable MCS karyotype with a low level of polyploidy
60. - freedom from oncogenicity or tumorigenicity by using *in-vivo* studies using the highest cell
61. passage that may be used for production
62. - purity of MCSs from extraneous bacteria, fungi, mycoplasma, and viruses
63. - implemented measures to lower TSE contamination risk (see Section C.3.5.1 *Purity tests*).

##### 3.2. Method of vaccine manufacturing

1. ~~The method of manufacture should be documented as the Outline of Production.~~
2. ~~2.2.1. Procedure~~
3. **3.2.1. LSD vaccine batch production**
4. ~~Vaccine batches are produced on an appropriate cell line such as MDBK.~~ As already mentioned in
5. the first paragraphs of Section C, all steps undertaken in the production of vaccine batches should
6. be described and documented in the Outline of Production. The production of LAV and IV against
7. LSD starts with the inoculation of the required number of working vials of seed virus ~~is~~ reconstituted
8. ~~with GMEM or other~~ in appropriate medium ~~and inoculated~~ onto a suitable primary or continuous cell
9. line grown in suspension or monolayer~~. Cells should be harvested after 4–8 days when they exhibit~~
10. ~~50–70% CPE for maximum~~ in the exponential growth phase. At the time highest viral ~~infectivity, or~~
11. ~~earlier if CPE is extensive and cells appear ready to detach. Techniques such as~~ loads are present,
12. sonication or repeated freeze–thawing ~~are~~ is used to release the intracellular virus from the
13. cytoplasm. The lysate may then be clarified using centrifugation to remove cellular debris ~~(for~~
14. ~~example by use of centrifugation at 600~~ ***~~g~~*** ~~for 20 minutes~~, with retention of the supernatant). ~~A second~~
15. ~~passage of the virus may be required to produce sufficient virus for a production batch.~~
16. An aliquot of the virus suspension is titrated to check the virus titre. For LAV, the virus-containing
17. suspension is diluted to attain the dose at which the vaccine candidate will be evaluated or to at least
18. the determined protective dose for approved vaccines and is then mixed with a suitable protectant
19. ~~such as an equal volume of sterile, chilled 5% lactalbumin hydrolysate and 10% sucrose (dissolved~~
20. in double-distilled water or appropriate balanced salt solution), and transferred to individually
21. ~~numbered~~ labelled bottles or bags for storage at low temperatures such as –80°C, or for freeze–
22. drying. A written record of all the procedures followed must be kept for all vaccine batches.
23. ~~2.2.2. Requirements for substrates and media~~
24. ~~The specification and source of all ingredients used in the manufacturing procedure should be~~
25. ~~documented and the freedom of extraneous agents (bacteria, fungi, mycoplasma and viruses) should~~
26. ~~be tested. The detailed testing procedure is described in Chapter 1.1.9. The use of antibiotics must~~
27. ~~meet the requirements of the licensing authority.~~
28. 2~~.2.3. In-process control~~
29. ~~i) Cells~~
30. ~~ii) Records of the source of the master cell stocks should be maintained. The highest and lowest~~
31. passage numbers of the cells that can be used for vaccine production must be indicated in the
32. Outline of the Production. The use of a continuous cell line (such as MDBK, etc.) is strongly
33. recommended, unless the virus strain only grows on primary cells. The key advantage of
34. continuous over primary cell lines is that there is less risk of introduction of extraneous agents.
35. ~~iii) Serum~~
36. ~~iv) Serum used in the growth or maintenance medium must be free from antibodies to capripoxvirus~~
37. and free from contamination with pestivirus or other viruses, extraneous bacteria, mycoplasma
38. ~~or fungi.~~
39. ~~v) Medium~~
40. ~~vi) Media must be sterile before use.~~
41. ~~vii) Virus~~
42. ~~viii) Seed virus and final vaccine must be titrated and pass the minimum release titre set by the~~
43. manufacturer. For example, the minimum recommended field dose of the South African
44. Neethling strain vaccines (Mathijs *et al.,* 2016) is log10 3.5 TCID50, although the minimum
45. protective dose is log10 2.0 TCID50. Capripoxvirus is highly susceptible to inactivation by sunlight
46. ~~and allowance should be made for loss of activity in the field.~~
47. ~~ix) The recommended field dose of the Romanian sheep pox vaccine for cattle is log10 2.5 sheep~~
48. infective doses (SID50), and the recommended dose for cattle of the RM65-adapted strain of
49. ~~Romanian sheep pox vaccine is log10 3 TCID50 (Coakley & Capstick, 1961).~~
50. 3.2.2. Inactivation process for inactivated LSD vaccines
51. Unlike LAV, inactivated LSD vaccines contain inactivated antigens in combination with adjuvants to
52. strengthen the induced immune response after administration. The vaccine evaluation process
53. described below needs to show the amount of antigen necessary to elicit a protective immune
54. response. Currently, literature data indicate that an inactivated vaccine originating from an LSDV
55. virus stock with titre 104 cell culture infectious dose50 (CCID50)/ml before inactivation can be sufficient
56. to induce an efficient immune response to prevent clinical disease, viremia and virus shedding after
57. challenge of young cattle (Wolf *et al.,* 2022)
58. To monitor the inactivation process and the level of antigen inactivation, samples are taken at regular
59. intervals during inactivation and titrated. Inactivation conditions and the length of initial and repeated
60. exposure should be documented in detail since one or more factors during the process could
61. influence the outcomes. The inactivation kinetics should reach a predefined target e.g. one remaining
62. infectious unit per million doses (1 × 10–6 infectious units/dose) as suggested by APHIS (2013). The
63. confirmatory testing of inactivation is performed on each vaccine lot and represents an important part
64. of the inactivation process monitoring. In addition to all the procedures mentioned above, the
65. inactivation procedure and tests demonstrating that antigen inactivation is complete and consistent
66. must additionally be documented in the Outline of Production.

##### 3.3. Vaccine safety

1. During the vaccine development process, vaccine safety must be evaluated in the target animal (target animal batch
2. safety test –TABST) to demonstrate the safety of the dose intended for registration. The animals used in the safety
3. testing should be representative (species, age and category [calves, heifers, bulls, cows.]) for all the animals for which
4. the vaccine is intended. Vaccinated and control groups are appropriately acclimatised, housed and managed in line
5. with animal welfare standards. Animal suffering has to be eliminated or reduced and euthanasia is recommended in
6. moribund animals.
7. Essential parameters to be evaluated in safety studies are local and systemic reactions to vaccination, including local
8. reactions at the site of administration, fever, effect on milk production, and induction of a ‘Neethling’ response. The
9. effect of the vaccine on reproduction needs to be evaluated where applicable.
10. A part of the safety evaluation of LAV and IV can be performed during the efficacy trials (see Section C.3.4 *Vaccine*
11. *efficacy*) by measuring local and systemic responses following vaccination and before challenge.
12. Guidelines for safety evaluation are provided by the European Medicine Agency (EMEA) in VICH GL44: TABST for
13. LAV and IV (EMEA, 2009). Safety aspects of LAV and IV against LSD to be evaluated are:
14. 3.3.1. Overdose test for LAV
15. Local and systemic responses should be measured following an overdose test whereby 10× the
16. maximum vaccine titre is administered. If the maximum vaccine titre is not specified, 10× the minimum
17. vaccine titre can be applied in multiple injection sites. Ideally, the 10× dose is dissolved in the 1×
18. dose volume of the adjuvants or diluent. Generally, eight animals per group should be used (EMEA,
19. 2009).
20. 3.3.2. One dose and repeat dose test
21. This aims to test the safety of the vaccine dose applied in the vaccination regime intended for
22. registration. LAV LSD vaccines require one dose per year, while inactivated LSD vaccines require a
23. booster dose in addition to the primary dose. The minimal recommended interval between
24. administrations is 14 days.
25. Generally, eight animals per group should be used unless otherwise justified (EMEA, 2009). For each
26. target species, the most sensitive breed, age and sex proposed on the label should be used.
27. Seronegative animals should be used. In cases where seronegative animals are not reasonably
28. available, alternatives should be justified.
29. 3.3.3. Reversion to virulence tests
30. Live attenuated vaccines inherently carry the risk of vaccine virus reverting to virulence when
31. repeated passages in a host species could occur due to shedding and transmission from vaccinated
32. animals to contact animals. LAV LSD vaccines should therefore be tested for non-reversion to
33. virulence by means of passage studies. Vaccine virus (MSV, not the finished vaccine) is inoculated
34. in a group of target animals of susceptible age via the natural route of infection or the route that is
35. most likely to result in infection. The vaccine virus is subsequently recovered from tissues or
36. excretions and is used directly to inoculate a further group of animals. After not less than four
37. passages (see chapter 1.1.8), i.e. use of a total of five groups of animals, the re-isolate must be fully
38. characterised, using the same procedures used to characterise the master seed virus.
39. 3.3.4. Environmental consideration
40. This includes the evaluation of the ability of LAV LSD vaccines to be shed, to spread and to infect
41. contact target and non-target animals, and to persist in the environment.
42. ~~2.2.4. Final product batch tests~~
43. ~~i) Sterility/purity~~
44. ~~ii) Vaccine samples must be tested for sterility/purity.~~ *~~Tests for sterility and freedom from~~*
45. *~~contamination of biological materials intended for veterinary use~~* ~~may be found in Chapter 1.1.9.~~
46. ~~iii) Safety and efficacy~~
47. ~~iv) The efficacy and safety studies should be demonstrated using statistically valid vaccination–~~
48. challenge studies using seronegative young LSDV susceptible dairy cattle breeds. The group
49. numbers recommended here can be varied if statistically justified. Fifteen cattle are placed in a
50. high containment level large animal unit and serum samples are collected. Five randomly
51. chosen vials of the freeze-dried vaccine are reconstituted in sterile PBS and pooled. Two cattle
52. are inoculated with 10 times the recommended field dose of the vaccine, and eight cattle are
53. inoculated with the recommended field dose. The remaining five cattle are unvaccinated control
54. animals. The animals are clinically examined daily and rectal temperatures are recorded. On
55. day 21 after vaccination, the animals are again serum sampled and challenged with a known
56. virulent capripoxvirus strain. The challenge virus solution should also be tested free from
57. extraneous viruses. The clinical response is recorded during the following 14 days. Animals in
58. the unvaccinated control group should develop the typical clinical signs of LSD, whereas there
59. should be no local or systemic reaction in the vaccinates other than a raised area in the skin at
60. the site of vaccination, which should disappear after 4 days. Serum samples are again collected
61. on day 30 after vaccination. The day 21 serum samples are examined for seroconversion to
62. selected viral diseases that could have contaminated the vaccine, and the days 0 and
63. 30 samples are compared to confirm the absence of antibody to pestivirus. Because of the
64. variable response in cattle to LSD challenge, generalised disease may not be seen in all of the
65. ~~unvaccinated control animals, although there should be a large local reaction.~~
66. ~~v) Once the efficacy of the particular strain being used for vaccine production has been determined~~
67. in terms of minimum dose required to provide immunity, it is not necessary to repeat this on the
68. ~~final product of each batch, provided the titre of virus present has been ascertained.~~
69. ~~vi) Batch potency~~
70. ~~vii) Potency tests in cattle must be undertaken for vaccine strains of capripoxvirus if the minimum~~
71. immunising dose is not known. This is usually carried out by comparing the titre of a virulent
72. challenge virus on the flanks of vaccinated and control animals. Following vaccination, the
73. flanks of at least three animals and three controls are shaved of hair. Log10 dilutions of the
74. challenge virus are prepared in sterile PBS and six dilutions are inoculated intradermally (0.1 ml
75. per inoculum) along the length of the flank; four replicates of each dilution are inoculated down
76. the flank. An oedematous swelling will develop at possibly all 24 inoculation sites on the control
77. animals, although preferably there will be little or no reaction at the four sites of the most dilute
78. inocula. The vaccinated animals may develop an initial hypersensitivity reaction at sites of
79. inoculation within 24 hours, which should quickly subside. Small areas of necrosis may develop
80. at the inoculation site of the most concentrated challenge virus. The titre of the challenge virus
81. is calculated for the vaccinated and control animals; a difference in titre >log10 2.5 is taken as
82. ~~evidence of protection.~~

##### 3.4. Vaccine efficacy

1. Data enclosed in the vaccine candidate dossier should support the efficacy of the vaccine in each animal species for
2. each vaccination regimen that is described in the product label recommendation. This includes studies regarding the
3. onset of protection when claims for onset are made and for the duration of immunity. Efficacy studies should be
4. conducted with the vaccine candidate that has been produced at the highest passage level permitted for vaccine
5. production as specified in the Outline of Production.
6. Efficacy (and safety) should be demonstrated in vaccination–challenge studies using representative (by species, age
7. and category) seronegative healthy animals for which the vaccine is intended and which are tested negative for
8. standard viral pathogens.
9. An example of a vaccination-challenge test set-up is outlined here. The group numbers mentioned can be varied if
10. statistically justified. Thirteen animals are placed in a high containment large animal unit and are divided into two
11. groups:
12. - single/repeated dose test group (*n*=8) – animals inoculated with the vaccine dose and route intended for
13. registration (in case of an IV against LSD, a booster dose should follow primary vaccination after minimum 14
14. days).
15. - control group (*n*=5) – non-vaccinated animals
16. Throughout the *in-vivo* study, all animals are clinically examined and rectal temperatures recorded. Blood, serum and
17. swab samples are regularly collected and subjected to laboratory testing. On day 21 after the vaccination with a LAV
18. or after the booster vaccination for an IV, the animals in both groups are challenged with a known virulent LSDV
19. strain. The challenge virus solution should be of known titre and tested free from extraneous viruses. Experience
20. obtained from previous animal experiments indicates that a dose of challenge virus between 104.0 and 106.5 TCID50
21. produces clinical disease in about half of the susceptible experimental cattle (;.Tuppurainen *et al*., 2021).
22. The clinical response following challenge is recorded over a period of 14 days. No clinical signs should occur in the
23. vaccinates, other than a local reaction at the site of inoculation. At least 1 animal in the unvaccinated control group
24. should develop the typical clinical signs of LSD. Although a generalised disease with skin nodules may not be seen
25. in all the unvaccinated control animals based on the knowledge that the outcome of a LSDV infection can range from
26. inapparent to severe, at the very least a large local reaction is to be expected.
27. Clinical and laboratory results will enable assessment of the safety and efficacy of the LSD vaccine candidate and
28. the induced immune responses. Serum samples collected at different time points during the trial can be examined to
29. study seroconversion against selected viral diseases that could have contaminated the vaccine.

##### ~~2.3. Requirements for regulatory approval~~

1. ~~2.3.1. Safety requirements~~
2. ~~i) Target and non-target animal safety~~
3. ii) The vaccine must be safe to use in all breeds of cattle for which it is intended, including young
4. ~~and pregnant animals. It must also be non-transmissible and remain attenuated after further~~
5. ~~tissue culture passage.~~
6. iii) Safety tests should be carried out on the final product of each batch as described in Section

701 ~~C.2.2.4.~~

1. ~~iv) Reversion-to-virulence for attenuated/live vaccines~~
2. v) The selected final vaccine should not revert to virulence during further passages in target
3. ~~animals.~~
4. ~~vi) Environmental consideration~~
5. vii) Attenuated vaccine should not be able to perpetuate autonomously in a cattle population.
6. ~~Strains of LSDV are not a hazard to human health.~~
7. ~~2.3.2. Efficacy requirements~~
8. ~~i) For animal production~~
9. The efficacy of the vaccine must be demonstrated in statistically valid vaccination challenge
10. experiments under laboratory conditions. The group numbers recommended here can be varied if
11. statistically justified. Fifteen cattle are placed in a high containment level large animal unit and serum
12. samples are collected. Five randomly chosen vials of the freeze-dried vaccine are reconstituted in
13. sterile PBS and pooled. Two cattle are inoculated with 10 times the field dose of the vaccine, eight
14. cattle are inoculated with the recommended field dose. The remaining five cattle are unvaccinated
15. control animals. The animals are clinically examined daily and rectal temperatures are recorded. On
16. day 21 after vaccination, the animals are again serum sampled and challenged with a known virulent
17. capripoxvirus strain using intravenous and intradermal inoculation (the challenge virus solution
18. should also be tested and shown to be free from extraneous viruses). The clinical response is
19. recorded during the following 14 days. Animals in the unvaccinated control group should develop the
20. typical clinical signs of LSD, whereas there should be no local or systemic reaction in the vaccinates
21. other than a raised area in the skin at the site of vaccination which should disappear after 4 days.
22. Serum samples are again collected on day 30 after vaccination. The day 21 serum samples are
23. examined for seroconversion to selected viral diseases that could have contaminated the vaccine,
24. and the days 0 and 30 samples are compared to confirm the absence of antibody to pestivirus.
25. Because of the variable response in cattle to challenge with LSDV, generalised disease may not be
26. ~~seen in all of the unvaccinated control animals, although there should be a large local reaction.~~
27. Once the potency of the particular strain being used for vaccine production has been determined in
28. terms of minimum dose required to provide immunity, it is not necessary to repeat this on the final
29. ~~product of each batch, provided the titre of virus present has been ascertained.~~
30. ~~ii) For control and eradication~~
31. ~~Vaccination is the only effective way to control LSD outbreaks in endemic countries and recent~~
32. ~~experiences of the disease in Eastern Europe and the Balkans suggests this is also true for outbreaks~~
33. in non-endemic countries. Unfortunately, currently no marker vaccines allowing a DIVA strategy are
34. ~~available, although to a limited extent PCR can be used for certain vaccines.~~
35. ~~The duration of immunity produced by LSDV vaccine strains is currently unknown.~~
36. ~~2.3.3. Stability~~
37. ~~All vaccines are initially given a shelf life of 24 months before expiry. Real-time stability studies are~~
38. ~~then conducted to confirm the appropriateness of the expiry date. Multiple batches of the vaccine~~
39. ~~should be re-titrated periodically throughout the shelf-life period to determine the vaccine stability.~~
40. ~~Properly freeze-dried preparations of LSDV vaccine, particularly those that include a protectant, such~~
41. ~~as sucrose and lactalbumin hydrolysate, are stable for over 25 years when stored at~~
42. ~~–20°C and for 2–4 years when stored at 4°C. There is evidence that they are stable at higher~~
43. ~~temperatures, but no long-term controlled experiments have been reported. No preservatives other~~
44. ~~than a protectant, such as sucrose and lactalbumin hydrolysate, are required for the freeze-dried~~
45. ~~preparation.~~

##### 3.5. Batch/serial tests before release for distribution

1. Quality tests on MSV and safety and efficacy tests on vaccine candidates are performed during the evaluation process
2. for new LSD vaccines. Once vaccines are approved to be used in the field, it remains important to verify the quality
3. of each vaccine batch produced. An independent batch quality control assessment may be warranted or requested
4. by national or international regulatory authorities.
5. 3.5.1. Purity test
6. Purity is defined by the absence of different contaminants (bacteria, fungi, mycoplasma, and other
7. viruses; see full details in chapter 1.1.9) in the vaccine and its associated diluent/adjuvants. Virus
8. isolation and bacterial culture tests can be used to show freedom from live competent replicating
9. microorganisms, but molecular methods are more rapid and sensitive, but positives can be caused
10. by genome fragments and incompetent replicating microorganisms.
11. Besides the contaminants mentioned above, manufacturers should demonstrate implemented
12. measures to minimise the risk of TSE contamination in ingredients of animal origin such as:
13. - all ingredients of animal origin in production facilities are from countries recognised as having the lowest
14. possible risk of bovine spongiform encephalopathy
15. - tissues or other substances used are themselves recognised as being of low or nil risk of containing TSE
16. agents
17. 3.5.2. Identity tests
18. In addition to identity tests performed on the MSV, the identity tests on final batches aim to
19. demonstrate the presence of only the selected capripoxvirus species and strain in the vaccine as
20. indicated in the Outline of Production and the absence of other strains or members of the genus and
21. any other viral contaminant that might arise during the production process. Identity testing could be
22. assured by using appropriate tests (e.g. PCRs, sanger sequencing, NGS).
23. 3.5.3. Potency tests
24. Standard requirements for potency tests can be found in CFR Title 9 part 113, in the European
25. Pharmacopoeia, and in this Terrestrial Manual.
26. **3.5.3.1. Live vaccines**
27. The potency of LAV against LSD can be measured by means of virus titration. The virus titre
28. must, as a rule, be sufficiently greater than that shown to be protective in the efficacy test
29. for the vaccine candidate. This will ensure that at any time prior to the expiry date, the titre
30. will be at least equal to the evaluated protective titre. The titres of currently available
31. commercial homologous LSD vaccines range between 103 and 104 infectious units/dose
32. (Tuppurainen *et al*., 2021).
33. **3.5.3.2. Inactivated LSD vaccines**
34. For inactivated LSD vaccines, potency tests are performed using vaccination–challenge
35. efficacy studies in animal hosts (see Section C.3.4. *Vaccine efficacy*).
36. 3.5.4. Safety/efficacy
37. Safety and efficacy testing is undertaken during the evaluation process of the vaccine candidate, and
38. also needs to be performed on a number of vaccine batches until robust data are generated in line
39. with international and national regulations. Afterwards, when using a seed lot system in combination
40. with strict implementation of GMP standards and depending on local regulations, TABST could be
41. waived as described in VICH50 and VICH55, providing the titer has been ascertained using potency
42. testing. Batches or serials are considered satisfactory if local and systemic reactions to vaccination
43. are in line with those described in the dossier of the vaccine candidate and product literature.
44. **3.5.4.1. Field safety/efficacy tests**
45. Field testing of two or more batches should be performed on all animal categories for which
46. the product is indicated before release of the product for general use (see chapter 1.1.8).
47. The aim of these studies is to demonstrate the safety and efficacy of the product under
48. normal field conditions of animal care and use in different geographical locations where
49. different factors may influence product performance. A protocol for safety/efficacy testing in
50. the field has to be developed with defined observation and recording procedures. However,
51. it is generally more difficult to obtain statistically significant data to demonstrate efficacy
52. under field conditions. Even when properly designed, field efficacy studies may be
53. inconclusive due to uncontrollable outside influences.
54. **3.5.4.2. Duration of Immunity**
55. The duration of immunity (DOI) following vaccination should be demonstrated via challenge
56. or the use of a validated serology test. Efficacy testing at the end of the claimed period of
57. protection should be conducted in each species for which the vaccine is indicated or the
58. manufacturer should indicate that the DOI for that species is not known. Likewise, the
59. manufacturer should demonstrate the effectiveness of the recommended booster regime in
60. line with these guidelines, usually by measuring the magnitude and kinetics of the
61. serological response observed.

#### ~~3. Vaccines based on biotechnology~~

1. ~~A new generation of capripox vaccines is being developed that uses the LSDV as a vector for the expression and delivery~~
2. ~~of immuno-protective proteins of other ruminant pathogens with the potential for providing dual protection (Boshra~~ *~~et al~~*~~.,~~
3. ~~2013; Wallace & Viljoen, 2005), as well as targeting putative immunomodulatory genes for inducing improved immune~~
4. ~~responses (Kara~~ *~~et al.,~~* ~~2018).~~
5. **4. Post-market studies**

##### 4.1. Stability

1. Stability testing shall be carried out as specified in Annex II of Regulation (EU) 2019/6 and in the Ph. Eur. 0062:
2. Vaccines for veterinary use, on not fewer than three representative batches providing this mimics the full-scale
3. production described in the application. At the end of shelf-life, sterility has to be re-evaluated using sterility testing
4. or by showing container closure integrity. Multiple batches of the vaccine should be re-titrated periodically throughout
5. the shelf-life period to determine the vaccine stability.

##### 4.2. Post-marketing surveillance

1. After release of a vaccine, its performance under field conditions should continue to be monitored by competent
2. authorities and by the manufacturer itself. Not all listed adverse effects may show up in the clinical trials performed
3. to assess safety and efficacy of the vaccine candidate due to the limited number of animals used. Post-marketing
4. surveillance studies can also provide information on vaccine efficacy when used in normal practice and husbandry
5. conditions, on duration of induced immunity, on ecotoxicity, etc.
6. First, a reliable reporting system should be in place to collect consumer complaints and notifications of adverse
7. reactions. Secondly, post-marketing surveillance should be established to investigate whether the reported
8. observations are related to the use of the product and to identify, at the earliest stage, any serious problem that may
9. be encountered from its use and that may affect its future uptake. Vaccinovigilance should be an on-going and integral
10. part of all regulatory programmes for LSD vaccines, especially for live vaccines.

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1. **NB:** There are WOAH Reference Laboratories for lumpy skin disease (please consult the WOAH Web site:
2. https://[www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).](http://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3))
3. Please contact WOAH Reference Laboratories for any further information on
4. diagnostic tests, reagents and vaccines for lumpy skin disease

###### **NB:** First adopted in 1989. Most recent updates adopted in 2021.