CHAPTER 2.7.14.

SHEEP POX AND GOAT POX

SUMMARY

Sheep pox and goat pox are viral diseases of sheep and goats characterised by fever, generalised papules or nodules, vesicles (rarely), internal lesions (particularly in the lungs), and death. Both diseases are caused by strains of capripoxvirus, all of which can infect sheep and goats. Although most of the strains examined cause more severe clinical disease in either sheep or goats, some strains have been isolated that are equally pathogenic in both species.

Capripox is endemic in Africa north of the Equator, the Middle East, Turkey, Iran, Iraq, Afghanistan, Pakistan, India, Nepal, parts of the People's Republic of China and Bangladesh. The most recent outbreaks occurred in Mongolia in 2008 and 2009, an isolated outbreak in Greece in 2008, and in Kazakhstan and Azerbaijan in 2009. Goat pox has been established in Vietnam since 2005. The first goat pox outbreak in Chinese Taipei occurred in 2008 and in 2010 the disease reoccurred and was declared endemic.

Identification of the agent: *Laboratory confirmation of capripox is most rapid using the polymerase chain reaction (PCR) method or by the identification of typical capripox virions using the transmission electron microscope in combination with a clinical history consistent with generalised capripox infection. The capripox virion is distinct from that of the other poxvirus commonly infecting sheep and goats – a parapoxvirus that causes orf or contagious pustular dermatitis. A precipitating antigen can be identified by an agar gel immunodiffusion test (AGID) using biopsy material, however due to low sensitivity and cross reactivity with parapoxvirus, this is no longer recommended. Capripoxvirus will grow on tissue culture of ovine, caprine or bovine origin, although field isolates may require up to 14 days to grow or require one or more additional tissue culture passage(s). The virus causes intracytoplasmic inclusions, clearly seen using haematoxylin and eosin staining. The antigen can also be detected in tissue culture using specific sera and immunoperoxidase or immunofluorescence techniques. Capripoxvirus antigen and inclusion bodies may be seen in stained cryostat or paraffin sections of biopsy or post-mortem lesion material.*

An antigen-detection enzyme-linked immunosorbent assay (ELISA) using a polyclonal detection serum raised against a recombinant immunodominant antigen of capripoxvirus has been developed. Genome detection by a conventional or a real-time polymerase chain reaction (PCR) method using capripoxvirus-specific primers has also been reported.

Serological tests: *The virus neutralisation test is the most specific serological test, but because immunity to capripox infection is predominantly cell mediated, the test is not sufficiently sensitive to identify animals that have had contact with the virus and developed only low levels of neutralising antibody. The AGID and indirect immunofluorescence tests are less specific due to cross-reactions with antibody to other poxviruses. Western blotting using the reaction between the P32 antigen of capripoxvirus with test sera is both sensitive and specific, but is expensive and difficult to carry out. The use of this antigen or some other appropriate antigens expressed by a suitable vector, in an ELISA offers the prospect of an acceptable and standardised serological test.*

Requirements for vaccines: *Live and inactivated vaccines have been used for the control of capripox. All strains of capripoxvirus so far examined share a major neutralisation site and will cross protect. Inactivated vaccines give, at best, only short-term immunity.*

A. INTRODUCTION

Sheep pox and goat pox (capripox) are endemic in central and North Africa, the Middle East, India, China (People's Rep. of), Vietnam and Chinese Taipei. Capripox is caused by strains of capripoxvirus and produces a characteristic clinical disease in fully susceptible breeds of sheep and goats and would usually be difficult to confuse with any other disease. In indigenous animals, generalised disease and mortality are less common, although they are seen where disease has been absent from an area or village for a period of time, when intensive husbandry methods are introduced, or in association with other disease agents, such as peste des petits ruminants virus or foot and mouth disease virus.

Capripox is a major constraint to the introduction of exotic breeds of sheep and goats, and to the development of intensive livestock production. Strains of capripoxvirus that cause lumpy skin disease (such as Neethling), are also found in cattle, but there is no evidence that these strains will naturally cause disease in sheep and goats. The geographical distribution of lumpy skin disease partially differs from that of sheep pox and goat pox.

Strains of capripoxvirus do pass between sheep and goats, although most cause more severe clinical disease in only one species; recombination also occurs between these strains, producing a spectrum showing intermediate host preferences and a range of virulence. Some strains are equally pathogenic in both sheep and goats. Capripox has the potential to spread and become established in countries outside its normal distribution. In 1983 it spread into Italy, in 1985 and 1989 into Cyprus, and in 1988 and numerous subsequent occasions into Greece, but did not become established in these countries. In 1984, however, it spread into Bangladesh where it has persisted. In 2005, an outbreak in goats in Vietnam indicated that capripox has a wider distribution than previously recognised.

The incubation period is between 8 and 13 days following contact between an infected and susceptible animals. It may be as short as 4 days following experimental infection by intradermal inoculation or mechanical transmission by insects. Some breeds of European sheep, such as Soay, may die of acute infection before the development of skin lesions. In other breeds there is an initial rise in rectal temperature to above 40°C, followed in 2–5 days by the development of, at first, macules – small circumscribed areas of hyperaemia, which are most obvious on unpigmented skin – and then of papules – hard swellings of between 0.5 and 1 cm in diameter – which may cover the body or be restricted to the groin, axilla and perineum. Papules may be covered by fluid-filled vesicles, but this is rare. A flat haemorrhagic form of capripox has been observed in some breeds of European goat, in which all the papules appear to coalesce over the body; this form is always fatal.

Within 24 hours of the appearance of generalised papules, affected animals develop rhinitis, conjunctivitis and enlargement of all the superficial lymph nodes, in particular the prescapular lymph nodes. Papules on the eyelids cause blepharitis of varying severity. As the papules on the mucous membranes of the eyes and nose ulcerate, so the discharge becomes mucopurulent, and the mucosae of the mouth, anus, and prepuce or vagina become necrotic. Breathing may become laboured and noisy due to pressure on the upper respiratory tract from the swollen retropharyngeal lymph nodes, due to the developing lung lesions.

If the affected animal does not die in this acute phase of the disease, the papules start to become necrotic from ischaemic necrosis following thrombi formation in the blood vessels at the base of the papule. In the following 5–10 days the papules form scabs, which persist for up to 6 weeks, leaving small scars. The skin lesions are susceptible to fly strike, and secondary pneumonia is common. Anorexia is not usual unless the mouth lesions physically interfere with feeding. Abortion is rare.

On post-mortem examination of the acutely infected animal, the skin lesions are often less obvious than on the live animal. The mucous membranes appear necrotic and all the body lymph nodes are enlarged and oedematous. Papules, which may be ulcerated, can usually be found on the abomasal mucosa, and sometimes on the wall of the rumen and large intestine, on the tongue, hard and soft palate, trachea and oesophagus. Pale areas of approximately 2 cm in diameter may occasionally be seen on the surface of the kidney and liver, and have been reported to be present in the testicles. Numerous hard lesions of up to 5 cm in diameter are commonly observed throughout the lungs, but particularly in the diaphragmatic lobes.

The clinical signs and post-mortem lesions vary considerably with breed of host and strain of capripoxvirus. Indigenous breeds are less susceptible and frequently show only a few lesions, which could be confused with insect bites or contagious pustular dermatitis. However, lambs that have lost their maternally derived immunity, animals that have been kept isolated and animals brought into endemic areas from isolated villages, particularly if they have been subjected to the stress of moving long distances and mixing with other sheep and goats, and their pathogens, can often be seen with generalised and sometimes fatal capripox. Invariably there is high mortality in unprotected imported breeds of sheep and goats following capripoxvirus infection. Capripox is not infectious to humans.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

• Sample collection, submission and preparation

Material for virus isolation and antigen detection should be collected by biopsy or at post-mortem from skin papules, 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. Samples for genome detection by polymerase chain reaction (PCR) may be collected when neutralising antibody is present. Buffy coat from blood collected into EDTA (ethylene diamine tetra-acetic acid) during the viraemic stage of capripox (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, for virus isolation from the buffy coat, should be collected in tubes containing anticoagulant, placed immediately on ice and processed as soon as possible. In practice, the blood 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 and dry scabs for virus isolation, antigen detection and genome detection should preferably 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 that the transport medium does not penetrate the central part of the biopsy, which should be used for virus isolation/detection.

Material for histology should be prepared by standard techniques and stained with haematoxylin and eosin (H&E). Lesion material for virus isolation and antigen detection are homogenised. The following is an example of one technique for homogenisation: The tissue is minced using sterile scissors and forceps, and then ground with a sterile pestle in a 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 (2.5 µg/ml) and neomycin (200 IU/ml). The homogenised 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 5–8 ml unclotted blood by centrifugation at 600 ***g*** for 15 minutes; the buffy coat is 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 of 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 using a Ficoll gradient.

a) Culture

Capripoxvirus will grow in tissue culture of bovine, ovine or caprine origin, although primary or secondary cultures of lamb testis (LT) or lamb kidney (LK) cells are considered to be the most susceptible, particularly those derived from a wool sheep breed. The following is an example of an isolation technique; Either 1 ml of buffy coat cell suspension or 1 ml of clarified biopsy preparation supernatant is inoculated on to a 25 cm^2 tissue culture flask of 90% confluent LT or LK cells, and supernatant is allowed to absorb for 1 hour at 37°C. 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 or LK cells and a flying cover-slip, or tissue culture microscope slides, are also infected.

The flasks should be examined daily for 7–14 days for evidence of cytopathic effect (CPE). Contaminated flasks should be discarded. 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 4 days after infection; over the following 4–6 days these expand to involve the whole cell sheet. If no CPE is apparent by day 7, the culture should be freeze–thawed three times, and clarified supernatant inoculated on to fresh LT or LK 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 indicative of poxvirus infection. Syncytia formation is not a feature of capripoxvirus infection. If the CPE is due to capripoxvirus infection of the cell culture, it can be prevented or delayed by inclusion of specific anti-capripoxvirus serum in the medium; this provides a presumptive identification of the agent.

Some strains of capripoxvirus have been adapted to grow on African green monkey kidney (Vero) cells, but these are not recommended for primary isolation.

- **Electron microscopy**

Material from the original suspension is prepared for transmission electron microscope examination, prior to centrifugation, by floating a 400-mesh hexagon electron microscope grid, with pileoform-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, no orthopoxvirus causes lesions in sheep and goats. However, capripoxvirus is distinguishable from the virions of parapoxvirus, that cause contagious pustular dermatitis, as they are smaller, oval in shape, and each is covered in a single continuous tubular element, which appears as striations over the virion.

- **Histology**

Following preparation, staining with H&E, and mounting of the formalin-fixed biopsy material, a number of sections should be examined by light microscopy. On histological examination, the most striking aspects of acute-stage skin lesions are a massive cellular infiltrate, vasculitis and oedema. Early lesions are characterised by marked perivascular cuffing. Initially infiltration is by macrophages, neutrophils and occasionally eosinophils, and as the lesion progresses, by more macrophages, lymphocytes and plasma cells. A characteristic feature of all capripoxvirus infections is the presence of variable numbers of 'sheep pox cells' in the dermis. These sheep pox cells can also occur in other organs where microscopic lesions of sheep and goat pox are present. These cells are large, stellate cells with eosinophilic, poorly defined intracytoplasmic inclusions and vacuolated nuclei. Vasculitis is accompanied by thrombosis and infarction, causing oedema and necrosis. Epidermal changes consist of acanthosis, parakeratosis and hyperkeratosis. Changes in other organs are similar, with a predominant cellular infiltration and vasculitis. Lesions in the upper respiratory tract are characterised by ulceration.

- **Animal inoculation**

Clarified biopsy preparation supernatant (see Section B.1.a Culture) may also be used for intradermal inoculation into susceptible lambs. These lambs should be examined daily for evidence of typical skin lesions of capripox.

b) Immunological methods

- **Fluorescent antibody tests**

Capripoxvirus antigen can also be identified on 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 sheep or goat sera is subject to high background colour and nonspecific reactions. However, a direct conjugate can be prepared from sera from convalescent sheep or goats or from rabbits hyperimmunised with purified capripoxvirus. Uninfected tissue culture should be included as a negative control because cross-reactions, due to antibodies to cell culture antigens, can cause problems. The fluorescent antibody tissue section technique has also been used on cryostat-prepared slides.

- **Agar gel immunodiffusion**

An agar gel immunodiffusion (AGID) test has been used for detecting the precipitating antigen of capripoxvirus, but has the disadvantage that this antigen is shared by parapoxvirus. Agarose (1%) is prepared in borate buffer, pH 8.6, dissolved by heating, and 2 ml is poured on to a glass microscope slide (76 × 26 mm). When the agar has solidified, wells are cut to give a six-well rosette around a central well. Each well is 5 mm in diameter, with a distance of 7 mm between the middle of the central well and the middle of each peripheral well. The wells are filled as follows: 18 µl of the lesion suspension is added to three of the peripheral wells, alternately with positive control antigen, and 18 µl of positive capripoxvirus control serum is added to the central well. The slides are placed in a humidified chamber at room temperature for 48 hours, and examined for visible precipitation lines using a light box. The test material is positive if a precipitation line develops with the control serum that is confluent with that produced by the positive control antigen. This test will not, however, distinguish between capripox infection and contagious pustular dermatitis (orf).

To prepare antigen for the AGID, one of two 125 cm^2 flasks of LT or LK cells is infected with capripoxvirus, and harvested when there is 90% CPE (8–12 days). The flask is freeze–thawed twice, and the cells are

shaken free of the flask. The contents are centrifuged at 4000 ***g*** for 15 minutes, most of the supernatant is decanted and stored, and the pellet is resuspended in the remaining supernatant. The cells should be lysed using an ultrasonic probe for approximately 60 seconds. This homogenate is then centrifuged as before, the resulting supernatant being pooled with that already collected. The pooled supernatant is added to an equal volume of saturated ammonium sulphate at pH 7.4 and left at 4°C for 1 hour. This solution is centrifuged at 4000 ***g*** for 15 minutes, and the precipitate is collected and resuspended in a small volume of 0.8% saline for use in the AGID test. The uninfected flask is processed in an identical manner throughout, to produce a tissue culture control antigen (Kitching *et al.*, 1986a).

- **Enzyme-linked immunosorbent assay**

Following the cloning of the highly antigenic capripoxvirus structural protein P32, it is possible to use expressed recombinant antigen for the production of diagnostic reagents, including the raising of P32 monospecific polyclonal antiserum and the production of monoclonal antibodies (MAbs) (Carn, 1995). Using hyperimmune rabbit antiserum raised by inoculation of rabbits with purified capripoxvirus, capripox antigen from biopsy suspensions or tissue culture supernatant can be trapped on an ELISA plate. The presence of the trapped antigen can then be detected using guinea-pig serum raised against the group-specific structural protein P32, commercial horseradish-peroxidase-conjugated rabbit anti-guinea-pig immunoglobulin and a chromogen/substrate solution.

c) Nucleic acid recognition methods

A conventional or a real-time PCR method can be used to detect the capripoxvirus genome in biopsy or tissue culture samples. Primers for the viral attachment protein gene and the viral fusion protein gene are specific for all the strains within the genus *Capripoxvirus* (Heine *et al.*, 1999; Ireland & Binepal, 1998). It is not possible to distinguish between strains of capripoxvirus from cattle, sheep or goats using serological techniques. Strains of virus can be identified using sequence and phylogenetic analysis (Le Goff *et al.*, 2009). Strains can also be characterised by comparing the genome fragments generated by *Hin*dIII digestion of their purified DNA (Black *et al.*, 1986; Kitching *et al.*, 1986). Using this techniques and by sequencing of the genome, differences between isolates from the different species have been identified (Hosamani *et al.*, 2004), but these are not consistent and there is evidence for the movement of strains between species and recombination between strains in the field (Gershon *et al.*, 1989).

The conventional gel-based PCR method described in Chapter 2.4.14 Lumpy skin disease can be used for the detection of sheep pox and goat pox viral DNA. Recently described real-time PCR methods are reported to be faster and have higher sensitivity (Balinsky *et al.*, 2008; Bowden *et al.*, 2008).

2. Serological tests

a) Virus neutralisation

A test serum can either be titrated against a constant titre of capripoxvirus (100 $TCID_{50}$ [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 $TCID_{50}$, 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 (Kitching & Taylor, 1985).

- **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 of row H.

iii) A reference strain of capripoxvirus, usually a vaccine strain known to grow well in tissue culture, with a titre of over $\log_{10}$ 6 $TCID_{50}$ per ml is diluted in Eagle's/HEPES in bijoux bottles to give a log dilution series of $\log_{10}$ 5.0; 4.0; 3.5; 3.0; 2.5; 2.0; 1.5 $TCID_{50}$ per ml (equivalent to $\log_{10}$ 3.7; 2.7; 2.2; 1.7; 1.2; 0.7; 0.2 $TCID_{50}$ 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 10^5 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 toxicity 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 starting at 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 according to 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 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.

b) Agar gel immunodiffusion

The AGID test cannot be recommended as a serological test for the diagnosis of capripox because of the cross-reaction with antibody to contagious pustular dermatitis virus, which is the main differential diagnosis. A consequence of this cross-reaction is many false-positive results.

c) 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 1/5, and positives are identified using an anti-sheep gamma-globulin conjugated with fluorescein isothiocyanate (Davies & Otema, 1978). Cross-reactions can occur with orf, bovine papular stomatitis virus and perhaps other poxviruses.

d) 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 (Chand *et al.*, 1994).

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 then 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. As an alternative to tissue culture antigen, it is possible to use purified virus or expressed recombinant P32 (Carn *et al.*, 1994; Heine *et al.*, 1999).

Molecular weight markers should be run concurrently with the protein samples. The separated proteins in the SDS/PAGE gel should then be transferred by electroblotting to a nitrocellulose membrane (NCM). After blotting, 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 can be 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 predetermined by titration. After further incubation at room temperature for 1.5 hours, the membrane is washed and a solution of diaminobenzidine tetrahydrochloride (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 (orf virus) will react with some of the capripoxvirus proteins, but not the 32 kDa protein that is specific for capripoxvirus.

e) Enzyme-linked immunosorbent assay

ELISAs for the detection of antibodies against capripox virus have been developed using the expressed structural P32 protein (Carn *et al.*, 1994; Heine *et al.*, 1999). More recently ELISA based on recombinant capripox antigens (Bowden *et al.*, 2009) and on inactivated, purified whole sheep pox virus (Babiuk *et al.,* 2009) have been described.

C. REQUIREMENTS FOR VACCINES

1. Background

a) Rationale and intended use of the product

A variety of attenuated live and inactivated capripoxvirus vaccines has been used to provide protection to sheep and goats against capripox (see refs Carn, 1993 and Kitching, 1983 for reviews). All strains of capripoxvirus of ovine, caprine or bovine origin examined so far share a major neutralising site, so that animals recovered from infection with one strain are resistant to infection with any other strain (Capstick, 1961). Consequently, it is possible to use a single strain of capripoxvirus to protect both sheep and goats against all field strains of virus, regardless of whether their origin was in Asia or Africa (Kitching *et al.*, 1986b; Kitching & Taylor, 1985).

There are two antigenic forms of capripoxvirus, the intact virion covered in short tubular elements, and the intact virion additionally covered in a host-cell-derived membrane. The latter is the form usually produced by the infected animal, whereas the former is that seen when virus is produced by freeze–thawing infected tissue culture. Dead vaccines produced from tissue culture are almost entirely naked virions, and when used as a vaccine do not stimulate immunity to the membrane-bound virion. This in part explains the poor success of inactivated vaccines. An additional factor is that inactivated vaccines are less effective than live, replicating vaccine virus in stimulating the cell-mediated immune response, which is the predominant protective response to poxvirus infection. Dead capripox vaccines provide, at best, only temporary protection. A number of strains of capripoxvirus have had widespread use as live vaccines (Davies & Mbugwa, 1985), for example the 0240 Kenya sheep and goat pox strain used in sheep and goats, the Romanian and RM-65 strains used mainly in sheep, and the Mysore and Gorgan strains used in goats. The protective dose depends on the vaccine strain used. Immunity in sheep and goats against capripox following vaccination with the 0240 strain lasts over a year, and will probably provide lifelong protection against lethal challenge. Similarly the Romanian strain gave protection for at least 30 months. The 0240 strain should not be used in *Bos taurus* breeds of cattle.

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. The documentation should include the standard operating procedures (SOP) for the method of manufacture and each step for the testing of cells and reagents used in the process, each batches and the final product.

a) Characteristics of the seed

i) Biological characteristics

A strain of capripoxvirus used for vaccine production must be accompanied by a history describing its origin and tissue culture or animal passage. It must be safe to use in all breeds of sheep and goats for which it is intended, including pregnant and young animals. It must 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. A quantity of master seed vaccine virus should be prepared and stored in order to provide a consistent working seed for regular vaccine production.

ii) 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/purity tests are described in Chapter 1.1.9. The master seed must also be safe and produce no clinical reaction in all breeds of sheep or goats when given by the recommended route and stimulate complete immunity to capripox in all breeds of sheep and goats for at least 1 year. The necessary safety and potency tests are described in paragraph C.2.b.iv Final product batch tests.

b) Method of manufacture

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

i) Procedure

Vaccine seed should be lyophilised and stored in 2 ml vials at –20°C. It may be stored wet at –20°C, but when wet, is more stable at –70°C or lower. The virus should be cultured in primary or secondary LT or LK cells of wool sheep origin for maximum yield. Vero cells may also be used with suitably adapted strains.

Vaccine batches are produced on fresh monolayers of secondary LT or primary LK cells. A vial of seed virus is reconstituted with GMEM or another appropriate medium and inoculated on to an LT or LK monolayer that has been previously washed with warm PBS, and allowed to adsorb for 15 minutes at 37°C before being overlaid with additional GMEM. After 4–6 days, there will be extensive (50–70%) CPE. The culture should be examined for any evidence of nonspecific CPE, medium cloudiness or change in medium pH. The culture is freeze–thawed three times, the suspension removed and centrifuged at 600 ***g*** for 20 minutes. A second passage may be required to produce sufficient virus for a production batch. Live vaccine may be produced on roller bottles.

The procedure is repeated and the harvests from individually numbered flasks are each mixed separately with an equal volume of sterile and chilled 5% lactalbumin hydrolysate and 10% sucrose, 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 for virus titration; 2 ml pools composed of 0.2 ml samples taken from ten bottles are used. A written record of all the procedures must be kept for all vaccine batches.

Inactivated vaccines are produced, usually from unattenuated field strains of capripoxvirus, grown in tissue culture as described above, inactivated with 0.03% formaldehyde, and mixed with an equal volume of alhydrogel as adjuvant. Formaldehyde is no longer considered to be a suitable inactivant for certain other viral vaccines because its mode of action cannot be guaranteed to be totally effective in inactivating all the live virus. This has not been fully investigated for capripoxvirus.

ii) Requirements for substrate and media

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

iii) In process controls

Cells: Cells should be obtained from the testis or kidney 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 three additional passages for further observation. They should be checked for the presence of noncytopathic strains of bovine virus diarrhoea or border disease viruses by immunofluorescence or immunoperoxidase techniques. If possible, cells 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 20×10^6 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 contamination with pestivirus or any other viruses, extraneous bacteria, mycoplasma or fungi.

Virus: Seed virus and final vaccine must be titrated in tissue culture tubes or microtitre plates. 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 tested negative for antibody to pestivirus to prevent the vaccine virus itself interfering with the test. The vaccine bulk can be held at –20°C or below until all sterility tests and titrations have been completed, at which time it should be freeze-dried in 1 ml aliquots in vials sufficient for 100 doses. The vaccine harvest diluted with lactalbumin hydrolysate and sucrose should have a minimum titre $\log_{10}$ 4.5 $TCID_{50}$ per ml after freeze-drying, equivalent to a field dose of $\log_{10}$ 2.5 $TCID_{50}$. A further titration is carried out on five randomly chosen vials of the freeze-dried preparation to confirm the titre.

iv) Final product batch tests

Sterility/purity

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

Safety and efficacy

The efficacy and safety studies should be demonstrated by statistically valid vaccination-challenge studies using seronegative young sheep and goats of known susceptibility to capripox virus. The procedure described is suitable for vaccine strains such as 0240 that are equally immunogenic in both sheep and goats. The choice of target animal should be adapted for strains with a more restricted host preference, such as Romanian and RM-65 strains for sheep or Mysore and Gorgan strains for goats.

Four sheep and four goats are placed in a high containment level animal unit and serum samples are collected. Five randomly chosen vials of the freeze-dried vaccine are reconstituted in 1 ml of sterile PBS each, and pooled. Overdose studies: one sheep and one goat are inoculated intradermally or subcutaneously with 0.2 ml of the 10 × concentrated vaccine. The remaining vaccine is diluted with sterile PBS and two sheep and two goats are inoculated intradermally or subcutaneously with 0.2 ml of the recommended field dose. The remaining sheep and goat are control animals. The animals are clinically examined daily and rectal temperatures are recorded. On day 21 after vaccination, the eight 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 capripox, whereas there should be no local or systemic reaction in the vaccinates other than a delayed-type hypersensitivity reaction, which will disappear within 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 day 0 and 30 samples are compared to confirm the absence of antibody to pestivirus.

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 due to the vaccine.

Batch potency

Potency tests must be undertaken if the minimum immunising dose of the virus strain 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 wool or hair. Log_{10} 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 macule/papule is measured at between 8 and 10 days post-challenge. The titre of the challenge virus is calculated for the vaccinated and control animals; a difference of $\log_{10}$ titre > 2.5 is taken as evidence of protection.

c) Requirements for authorisation

i) Safety requirements

Target and non-target animal safety

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

Safety tests should be carried out on the final product of each batch as described in paragraph C.2.b.iv Final product batch tests.

The safety of the vaccine in non-target animals must have been demonstrated using mice and guinea-pigs as described in paragraph C.2.b.iv Final product batch tests. There should be no evidence of pathology caused by the vaccine.

Reversion-to-virulence for attenuated/live vaccines

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

Environmental consideration

Attenuated vaccine should not be able to perpetuate autonomously in a cattle, sheep or goat populations. Vaccines using the 0240 strain should not be used in *Bos taurus* breeds. Strains of capripoxvirus are not a hazard to human health. There are no precautions other than those described above for sterility and freedom from adventitious agents.

ii) Efficacy requirements

For animal production

The efficacy of the vaccine must be demonstrated in vaccination challenge experiment under laboratory conditions. As described in Section C.2.b.iv Final product batch tests.

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.

For control and eradication

Vaccination is the only effective way to control the sheep pox and goat pox 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 of sheep or goats with the 0240 strain lasts over 1 year, and protection against generalised infection following intradermal challenge lasts at least 3 years and is effective lifelong. The duration of immunity produced by other vaccine strains should be ascertained in both sheep and goats by undertaking controlled trials in an environment in which there is no possibility of field strains of capripoxvirus confusing the results. The inactivated vaccines provide immunity for less than 1 year, and for the reasons given at the beginning of this section, may not give immunity to the form of capripoxvirus usually associated with natural transmission.

ii) 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 capripox 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. The inactivated vaccines must be stored at 4°C, and their shelf-life is usually given as 1 year.

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) Vaccines available and their advantages

Currently, no recombinant vaccines for capripoxviruses are commercially available. However, a new generation of capripox vaccines is being developed that uses the capripoxvirus genome as a vector for the genes of other ruminant pathogens, for instance genes of rinderpest and peste des petits ruminants (PPR) viruses. The recombinant vaccine will provide protection against capripox, rinderpest and PPR in a single vaccination (Berhe *et al.*, 2003).

b) Special requirements for biotechological vaccines, if any

Not applicable.

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NB: There are OIE Reference Laboratories for sheep pox and goat pox (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).