SECTION 3.6.

# LAGOMORPHA

CHAPTER 3.6.1.

# MYXOMATOSIS

## SUMMARY


***Definition of the disease:*** *Myxomatosis is a lethal, generalised viral disease of the European rabbit* (Oryctolagus cuniculus) *caused by the* Myxoma *virus* (*MYXV*)*, a member of the* Poxviridae *family. The natural hosts are two leporides:* Sylvilagus brasiliensis *in South America* (*South American strains*) *and* S. bachmani (*Californian strains*) *in California, USA. However, following deliberate introductions into Australia and Europe as a biological control for wild European rabbits, MYXV now has a worldwide distribution, is endemic in wild European rabbit populations, and can spill over into farmed, laboratory, and pet rabbits. To date, myxomatosis is still the main infection threat for the rabbit industries.*

***Description of the disease:*** *Myxomatosis is essentially a disease of European rabbits. Brown hares* (Lepus europaeus) *are susceptible to MYXV infection, but rarely develop generalised disease.* Sylvilagus *spp. are quite resistant and may act as healthy carriers. There is no health risk to humans. Two forms of the disease are observed in rabbits: the nodular* (*classical*) *form, characterised by gross myxomatous skin lesions, and the amyxomatous* (*respiratory*) *form, in which signs are mainly respiratory, skin nodules being few and small. The nodular form is caused by virulent MYXV strains, is naturally transmitted by biting insects, especially during summer time, and is mainly observed in wild and pet rabbits and in small-scale rabbitries. During MYXV infection the copious viral immunomodulatory proteins progressively induce the collapse of the host immune system. This favours bacterial infections in the respiratory tract that greatly contribute to the death of the animal. MYXV strains with different degrees of pathogenicity have arisen since the introduction of MYXV into rabbit populations and these circulate in the field. Mild and attenuated strains cause the amyxomatous* (*respiratory*) *form of the disease especially in farmed animals. Wild rabbits act as reservoirs while mosquitoes and fleas can transmit the virus to domestic rabbits, but in the case of close proximity between rabbits* (*i.e. in farmed animals*)*, virus may also be transmitted by direct contact. Introduced semen may also pose a risk. There is no age or sex predilection.*

***Identification of the agent:*** *The diagnosis of myxomatosis, regardless of its clinical form, depends on the isolation and identification of the virus or the demonstration of its antigens. When skin lesions are present on a dead rabbit, the viral antigen may be demonstrated by several rapid diagnostic methods such as agar gel immunodiffusion* (*AGID*)*, negative-staining electron microscopy* (*nsEM*)*, fluorescent antibody test* (*FAT*)*, polymerase chain reaction* (*PCR*)*, and histopathology. Monolayer cell cultures of rabbit kidney inoculated with lesion material will show the characteristic cytopathic effects of poxviruses. The presence of virus can be confirmed by immunoperoxidase monolayer assay, FAT, PCR and nsEM.*

***Serological tests****: The presence of an overt humoral immune response facilitates a retrospective diagnosis of the disease, and can provide an indication of the prevalence of infection in a rabbit population. Serology could be also used to evaluate vaccination even if there is not a direct correlation between the anti-MYXV titres and the degree of protection of animals from the disease. Identification and titration of specific antibodies arising from natural infection or from immunisation*

*is mainly done by enzyme-linked immunosorbent assay. IFA and AGID may be used but are less sensitive.*

***Requirements for vaccines:*** *Modified live virus vaccines prepared from fibroma virus or modified* Myxoma *virus strains are available for immunisation of rabbits.*

## A. INTRODUCTION

Myxomatosis is a major viral disease of wild and domestic European rabbits (*Oryctolagus cuniculus*) caused by the *Myxoma* virus (MYXV), a poxvirus (family *Poxviridae*; subfamily *Chordopoxvirinae*; genus *Leporipoxvirus*) first isolated from a colony of laboratory rabbits in Uruguay in 1898. The MYXV DNA encodes about 170 genes among which approximately 70 encode immunomodulatory and host interactive factors that are involved in subverting the host immune system and other anti-viral responses.

The natural hosts are two leporides: *Sylvilagus brasiliensis* in South America (South American strains) and *S. bachmani* (Californian strains) in California, USA (Fenner, 1994) in which the viral strains produce only a benign fibroma. Following deliberate introductions into Australia and Europe as a biological control for wild European rabbits, MYXV now has a worldwide distribution, is endemic in wild European rabbit populations, and can spill over into farmed, laboratory and pet rabbits (Fenner & Fantini, 1999). European hares may rarely develop generalised disease (Fenner & Ratcliffe, 1965). Wild rabbits act as reservoirs, and insects (mainly mosquitoes and fleas but also midges and lice) can transmit the virus to domestic rabbits. Where there is close proximity between rabbits (i.e. in farmed animals), virus may also be transmitted by direct contact. MYXV is shed in ocular and nasal secretions or from skin lesions and it is also potentially present in semen and genital secretions. There is no age or sex predilection.

Two forms of the disease are observed: the nodular (classical) form and the amyxomatous (respiratory) form. Nodular myxomatosis is naturally transmitted by biting insects and mainly observed in wild and pet rabbits and in small-scale rabbitries. It is characterised by florid skin lesions and severe immune dysfunction, accompanied by supervening bacterial infections of the respiratory tract. Prototype strains of virus deriving from the Australian and European outbreaks have been designated that characterise the various virulence grades (from grade I to grade V) as determined in laboratory rabbits (Fenner & Ratcliffe, 1965). After infection with a grade I (the most virulent) strain, the first sign of infection is a lump at the site of infection, which increases in size and usually becomes protuberant and ulcerates. An acute blepharo-conjunctivitis and an oedematous swelling of the genital area gradually develop. The secondary skin lesions appear on about the sixth or the seventh day (Fenner, 1994). Death usually occurs between the eighth and fifteenth day post-infection. After infection with grade II to V strains, the clinical signs are usually the same but they evolve more slowly and are less severe. When animals survive, the lesions progressively heal. The mortality rate fluctuates between 20 and 100%, according to the grade of virulence of the viral strain. Secondary bacterial infections (in particular *Pasteurella* sp. and *Bordetella* sp.) of the conjunctivae, upper respiratory tract, and lungs are typical in rabbits that survive longer than 10–14 days after infection and may be the major cause of death in rabbits infected with subacute strains of MYXV.

The clinical signs of amyxomatous myxomatosis are mainly respiratory: it has fewer, smaller, cutaneous lesions than the nodular type, although many of the clinical signs are typical of myxomatosis such as the cutaneous lesion at the inoculation site, perineal oedema, swollen eyelids, blepharoconjunctivitis and rhinitis. The amyxomatous form is regarded as more significant for farmed rabbits. The genetic basis for the amyxomatous phenotype has not been defined but it has been suggested that the amyxomatous form represents an adaptation to contact transmission in the absence of vectors, presumably via respiratory and conjunctival secretions, as direct contact is needed for transmission. The virulence of amyxomatous viruses appears to depend on the presence of bacterial pathogens such as *Pasturella multocida* (Marlier *et al.,* 2000). So far, this form is commonly reported in European countries with substantial rabbit meat production (e.g. France, Spain, Belgium, Italy).

There is no known risk of human infection with MYXV. Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities.*

Clinical signs of classic myxomatosis are fairly clear-cut, although bacterial upper respiratory tract infections and bacterial conjunctivitis/keratoconjunctivitis can cause confusion and misdiagnosis. Shope fibroma virus (SFV) produces a simple fibromatous local lesion that should be distinguished from MYXV.

## B. DIAGNOSTIC TECHNIQUES

As the signs of the disease become less distinct with the attenuation of virus strains, the submission of samples for laboratory diagnosis becomes more important. Moreover, the expression of ectodermotropism is reduced for

amyxomatous MYXV strains, so that the clinical diagnosis of the amyxomatous form is clearly more difficult than for the classical one. The different techniques available vary in their ability to detect MYXV in typical myxomatous lesions, oedema of the eyelids or genital oedema. Nevertheless, the diagnosis of attenuated typical myxomatosis or of atypical (amyxomatous) forms may require the isolation of the virus by inoculation of sensitive cell lines such as the RK-13 (rabbit kidney) cell line and identification of the virus by immunological methods. In both cases, the agent can also be more easily and quickly identified by demonstration of MYXV nucleic acid. The use of molecular techniques for diagnosis has progressively increased in recent years. These methods can reveal subclinical infection (e.g. by testing conjunctival swabs) and permit the differentiation of vaccine from wild field strains (Cavadini *et al.,* 2010).

***Table 1. Test methods available and their purpose***

| Method | Purpose | | | | | |
|---|---|---|---|---|---|---|
| | Population freedom from infection | Individual animal freedom from infection prior to movement | Contribute to eradication policies | Confirmation of clinical cases | Prevalence of infection – surveillance | Immune status in individual animals or populations post-vaccination |
| **Agent identification[1]** | | | | | | |
| **nsEM** | – | – | – | ++ | – | – |
| **Histopathogy and immunostaining** | – | – | – | ++ | – | – |
| **Virus isolation (cell culture)** | – | + | – | +++ | – | – |
| **AGID** | + | + | + | – | – | – |
| **FAT** | + | ++ | – | + | – | – |
| **IPMA** | + | + | – | ++ | – | – |
| **PCR** | ++ | +++ | ++ | ++ | – | – |
| **Detection of immune response** | | | | | | |
| **I-ELISA** | ++ | +++ | +++ | ++ | +++ | +++ |
| **C-ELISA** | ++ | +++ | +++ | ++ | +++ | +++ |
| **IFA** | + | + | + | – | ++ | ++ |
| **AGID** | + | + | + | – | + | ++ |

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.
Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
nsEM = negative staining electron microscopy; AGID = Agar gel immunodiffusion; FAT = fluorescent antibody test; IPMA = immunoperoxidase monlayer assay; PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay (I = indirect; C = competitive); IFA = indirect fluorescent antibody test.

## 1. Identification of the agent

In the case of the classical form of the disease, MYXV identification may be attempted from skin lesions (myxoma), eyelids, genital mucosa and internal organs (lungs, liver, spleen, kidney etc.). Myxomas are excised with scissors and separated from the epidermis and superficial dermis. Tissue samples (nodular and skin tissues,

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

portion of organs and mucosal scrapings) are washed with phosphate-buffered saline (PBS) with antibiotics as defined below and ground or mechanically homogenised at a dilution rate of 1 g tissue/4.5–9.0 ml of PBS or sterile distilled water ($dH_2O$). Cells are disrupted by two freeze–thaw cycles, or by ultrasonication to liberate virions and viral antigens. This suspension is centrifuged for 5–10 minutes at 1500 ***g***. The supernatant fluid is used for the tests.

In the case of the amyxomatous respiratory form of the disease, nasal and conjunctival swabs may be collected for viral identification. Swabs are put in a tube containing approximately 0.3 ml of sterile $dH_2O$ and soaked for 10–15 minutes, scraping any remaining specimen off the cotton swab directly into the $dH_2O$ with a wooden applicator stick.

### 1.1. Electron microscopy

Negative-staining electron microscopy (nsEM) can be applied to a portion of skin lesion (myxomas), eyelid, genital mucosa as well as on conjunctival and nasal swabs and lungs. The technique (drop method) is simple and rapid to perform, giving results in 1 hour.

A drop of the tissue suspension is laid in a watch glass and a plastic/carbon-coated 200/400-mesh copper grid (plastic-side down) is placed on drop and let absorbed for 10 minutes. Any excess liquid is then removed with filter paper and the grid is transferred on a drop of stain for approximately 30 seconds. Either a 2% aqueous solution of ammonium molybdate, pH 7.0, or 2% phosphotungstic acid (PTA), pH 7.0, may be used for staining. The excess liquid is removed with filter paper and the grid is ready for the EM examination. In a positive case, typical poxvirus particles can be seen, however MYXV cannot be distinguished from SFV using this method.

### 1.2. Histopathology

Histopathology of cutaneous lesions, fixed in 10% buffered formalin and embedded in paraffin, shows that the large lumps found in the skin are mainly due to an accumulation of mucinous material with destruction of the connective tissue architecture in the dermis rather than to an intense cellular proliferation (Marcato & Rosmini, 1986). The derma and epidermis are invaded by granulocytes and enlarged, stellate, reticulo-endothelial cells with a large nucleus and abundant cytoplasm, called “myxoma cells”. These cells destroy the endothelium of small vessels causing extravasation of red cells; they also replicate in the spleen and lymph nodes causing complete loss of lymphocytes from both B-cell and T-cell zones. After the viraemic phase, the virus spreads throughout the body and causes genital and visceral lesions, mainly congestive with vascular damage. In the lung the lesions are of variable intensity and the characteristic epidermal lesions are also observed in the bronchial epithelium (Joubert, 1973). Microscopic lesions may vary according to the virulence of strains and the type of animals i.e. in wild vs laboratory rabbits (Best *et al.,* 2000).

Fixed tissues can be also immunostained using an avidin–biotin complex (ABC) peroxidase method. The sections are first deparaffinised in xylene and alcohol, counter-stained with haematoxylin for 1 minute and rinsed in tap water. They are then put in a methanol bath containing 3% $H_2O_2$ and washed in PBS three times for 5 minutes each. To limit background interference caused by nonspecific antibody binding, the samples are incubated with normal rabbit serum for 1 hour at room temperature prior to the addition of biotin. The slides are incubated overnight in a humid chamber at room temperature with biotinylated anti-MYXV serum or monoclonal antibodies (MAbs), are washed as before and incubated again for 30 minutes at 37°C with an ABC peroxidase. The slides are then washed three times. Amino-ethyl-carbazole is used as substrate. Finally, the slides are rinsed in tap water and mounted.

### 1.3. *In-vitro* culture – cell culture

Isolation of the virus in cell culture can be accomplished using primary cultures of rabbit kidney (RK) cells, or with established cell lines, such as RK-13 and SIRC (Statens Seruminstitut rabbit cornea) but also other mammalian cell lines such as Vero (African green monkey kidney) and BGMK (buffalo green monkey kidney), in minimal essential medium (MEM) containing 2% calf serum, 300 international units (IU)/ml penicillin; 300 µg/ml streptomycin; 100 µg/ml gentamycin; 50 IU/ml nystatin (mycostatin); and 5 µg/ml amphotericin (fungizone). The inoculum consists of the supernatant fluid from a homogenised lesion or oculo-respiratory discharge (including conjunctival swab prepared as described above) in MEM with 2% calf serum and antibiotics. This is removed from the cell layer after 2 hours. The cell layer is washed in a small volume of medium and then replenished with maintenance medium (MEM). The cultures are incubated at 37°C in a 5% $CO_2$ atmosphere.

A cytopathic effect (CPE) typical of poxviruses (Joubert, 1973) usually develops after 24–48 hours, but with some strains, depending on their virulence, it may take up to 7 days for CPE to be observed.

According to the viral strain, groups of cells with a confluent cytoplasm form syncytia that vary in size from 2 to 50 or even 100 nuclei together. The nuclei of some cells change, the chromatin forming basophilic aggregations that vary in size and number and give the culture a leopard-skin appearance. Eosinophilic intracytoplasmic inclusions remain discrete, if present at all. Affected cells round up, contract and become pyknotic. They then lyse and become detached from the glass or plastic support. Later, all cells are affected and the cell monolayer detaches completely.

SFV at first produces well-defined voluminous masses of rounded cells, which proliferate and pile up (Joubert, 1973). At the edge, cells just becoming infected show discrete nuclear changes and acidophilic cytoplasmic inclusions that are numerous at an early stage. The cell layer is destroyed after several days.

In addition to the observation of CPE, different methods can be used to confirm the viral isolation on cell culture. Including nsEM (see Section B.1.1), FAT (see Section B.1.5.2), IPMA (see Section B.1.5.4) and PCR (see Section B.1.6).

## 1.4. *In-vivo* culture

### 1.4.1. Embryonated eggs

MYXV and SFV can be cultured on the chorioallantoic membrane of embryonated chicken eggs. Eleven-day-old eggs are inoculated chorioallantoically and incubated at 35°C for 3 days. In case of virus growth, specific pocks may be observed under the microscope after removing and washing the membrane.

### 1.4.2. Animal inoculation

Rabbit intradermal inoculation is not recommended as a diagnostic tool. However it may be used where it is necessary to characterise the pathogenicity (virulence grade, classical or amyxomatous forms) or to distinguish SFV from MYXV. Rabbits should be of a domestic breed, weighing approximately 2 kg, unvaccinated and previously tested for the absence of antibodies (Joubert, 1973).

The inoculum may be the supernatant fluid from a homogenised lesion (with antibiotics) or the product of a cell culture. Between 0.1 and 0.2 ml is administered intradermally behind the ear or into the dorso-lumbar region, which has previously been depilated. The inoculum may be assayed by injecting serial dilutions in saline buffer at one site for each dilution. A primary lesion will appear at the sites within 2–5 days, followed by conjunctivitis. Using five sites for each dilution allows a 50% infective dose ($ID_{50}$) to be obtained. If the animal survives, the disease can be confirmed serologically after 15 days.

## 1.5. Antigen detection – immunolabelling techniques

### 1.5.1. Agar gel immunodiffusion (AGID)

Agar gel immunodiffusion (AGID) (Sobey *et al.,* 1966) is qualitative and can detect antigen or antibody. AGID tests are simple and rapid to perform – results can be obtained within 24 hours. Agar plates are prepared with Noble agar (0.6 g), ethylene diamine tetra-acetic acid (EDTA) (2.5 g), sodium chloride (4.5 g), and distilled water (500 ml) containing thiomersal (merthiolate) at 1/100,000 dilution. Standard antiserum (see below 2.c.), and the test sample are placed in opposing wells that are 6 mm in diameter and 5 mm apart. Another technique is to deposit a small portion of the lesion directly into the agar, 5 mm away from a filter paper disk impregnated with the antiserum. A number of lines of precipitation, usually up to three, appear within 48 hours, indicating the presence of myxoma viral antigens. Only one line forms in the presence of heterologous reactions with SFV.

### 1.5.2. Fluorescent antibody test (FAT)

Tissue cryosections fixed in methanol can be directly immunostained by incubation for 1 hour with fluorescein-conjugated anti-MYXV serum or MAbs. Specific fluorescence can be detected in the cutaneous lesions, in eyelids, lungs, spleen, liver, kidney or genital mucosa. An *in-vivo* DIF test on impressions of cornea, eyelid and conjunctival cells has been described (DIF-ET) (Cancellotti *et al.,* 1986). The DIF-ET is performed by a glass slide gently pressed on the surface of the eye. In this manner the cornea, eyelid and conjunctival cells adhere to the glass and can be stained and observed.

### 1.5.3. Indirect fluorescent antibody test (IFAT)

Indirect fluorescent antibody tests (IFAT) can be applied to cultures from 24 hours onwards. IFAT reveal intracytoplasmic multiplication of virus, but cannot distinguish MYXV from SFV. The inoculation of chicken embryo cells (trypsinised at day 11 of egg incubation) does not result in CPE, but it is useful for detecting the viral antigens by IFAT.

### 1.5.4. Immunoperoxidase monolayer assay (IPMA)

The test can be used to identify MYXV-infected cells. RK-13 cells are seeded on a six-well plate (see Section 2.1.1.ii). When cells are ready, inoculate the samples, one for each well, and incubate the plate as described at Section B.2.3.1. Fix the cells using a solution of cold 80% acetone for 5–10 minutes, then air dry. Incubate the cells with PBS with 0.3% $H_2O_2$ for 10 minutes at room temperature to quench the endogenous peroxide. Wash in PBS twice for 5 minutes each time. Incubate for 1 hour at 37°C with pre-titrated MYXV rabbit immune serum or specific MAbs in PBS containing 1% BSA. Wash in PBS three times for 5 minutes each. Incubate for 1 hour at 37°C with pre-titrated IgG anti-IgG rabbit (or mouse) horse radish peroxide conjugate. Wash in PBS four times for 5 minutes each. Stain with diaminobenzidine (DAB) solution (0.05% DAB, 50 mm Tris/HCl pH 7.4, 0.01% $H_2O_2$ (freshly prepared) 10 minutes at room temperature. Wash with running tap water for 3 minutes. Infected cells are clearly visible under an inverted microscope as brown stained cells.

## 1.6. Molecular methods – detection of nucleic acid

Polymerase chain reaction (PCR) (Cavadini *et al.,* 2010) or real-time PCR (Albini *et al.,* 2012; Belsham *et al.,* 2010; Duarte *et al.*, 2013), can be used to amplify genome fragments of MYXV in diagnostic material including eyelid, ear and nasal myxomas, crusts and/or lung lesions, nasal and conjunctival swabs or semen.

PCR and PCR-RFLPs (restriction fragment length polymorphism) can also be used to detect vaccine strains (Borghi strain and SG33 strains) (Camus-Bouclainville *et al.,* 2011; Cavadini *et al.,* 2010).

Purification of total DNA is a prerequisite for achieving optimal sensitivity. Various purification methods are commercially available and suitable for the assay. Special precautions should be taken during all steps to minimise the risk of contamination.

The procedures described here in detail are modified from those reported by Cavadini *et al.* (2010). The PCR assay consists of three successive procedures: (1) DNA extraction from the test or control sample followed by (2) PCR amplification and (3) detection of the PCR products by agarose gel electrophoresis.

### 1.6.1. Extraction of viral DNA

i) Homogenise ~1 g of sample (eyelid, ear and nasal myxomas, crusts and/or lung lesions) in 9 ml of PBS1X using manual or electric homogeniser, spin for 15 minutes at 4°C at low speed ~2000 ***g***.

ii) 100 µl supernatant is added to 100 µl lysis buffer (50 mM Tris/HCl, pH 8, 100 mM $Na_2$ EDTA, 100 mM NaCl, 0.5% sodium dodecyl sulphate [SDS]) plus 12 µl of proteinase K (stock 20 mg/ml, final concentration 1.2 mg/ml) and incubated for 2 hours at 45°C (modified by Stuart, 2004)

iii) To inactivate the proteinase K, the homogenate is denatured for 10 minutes at 94°C and centrifuged at 12,000 ***g*** for 1 minute.

iv) The resulting supernatant is transferred to a new tube and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) is added, the mixture is vortexed, centrifuged for 10 minutes at 12,000 ***g*** and the upper phase transferred to a new tube.

v) Tto precipitate the DNA, 0.1 volume of 3M sodium acetate (pH 5.2) and two volumes of absolute ethanol are added and the mixture incubated at –20°C overnight or for shorter periods at –80°C (e.g. 20–30 minutes).

vi) Recover the precipitated DNA by centrifugation at 12,000 ***g*** for 5–15 minutes at 4°C.

vii) Remove the ethanol with care and wash the pellet with 1 ml of 70% ethanol (v/v).

viii) The dried DNA may be resuspended in 1 ml of TE buffer (10 mM Tris/Cl, pH 7.5, 1 mM EDTA) and stored at 4°C for further manipulation or at –20°C for long-term storage.

ix) For each session a negative control (100 µl of PBS) and a positive control (100 µl of homogenate from a positive rabbit) should be added.

### 1.6.2. PCR amplification and detection of *Myxoma* virus on agarose gel

i) Extracted DNA is amplified by PCR using primers:

M071-F: 5'-ACC-CGC-CAA-GAA-CCA-CAG-TAG-T-3' (67,229nt -67,250nt)

M071-R: 5'-TAA-CGC-GAG-GAA-TAT-CCT-GTA-CCA-3' (67,700nt -67,677nt) (Cavadini *et al.,* 2010).

ii) The PCR amplification is performed in 25 µl and the procedure and conditions are summarised in Tables 2 and 3.

iii) For each PCR reaction a negative control (water DNase free) and a positive control (5–10 ng of DNA previously extracted and tested from a positive rabbit) should be added.

***Table 2.*** *PCR reagents*

| Reagents | Final concentration | Volumes |
|---|---|---|
| DNA | | 5–10 ng |
| Buffer 5× | 1× | 5 µl |
| Primer-F (20 pmol/µl) | 0.4 pmol/µl | 0.5 µl |
| Primer-R (20 pmol/µl) | 0.4 pmol/µl | 0.5 µl |
| dNTPs (2.5 mM each) | 0.2 mM | 2 µl |
| BSA (1 mg/ml) | 0.1 mg/ml | 2.5 µl |
| Taq 5U/µl | 0.04 U/µl | 0.2 µl |
| $H_2O$ DNase free | | XX[2] |
| Final volume | | 25 µl |

***Table 3.*** *PCR-thermal profile*

| Steps | Temperature | Time | No. cycles |
|---|---|---|---|
| Denaturation | 95°C | 5 minutes | 1 |
| Denaturation | 95°C | 30 seconds | 40 |
| Annealing | 60°C | 30 seconds | |
| Extension | 72°C | 40 seconds | |
| Extension | 72°C | 7 minutes | 1 |
| | 4°C | ∞ | 1 |

iv) At the end of amplification 10 µl of the PCR reaction are analysed on 2% agarose gel and a positive result is indicated by the presence of a 471 bp band corresponding to a portion of M071L gene target in the positive control. If band appears in the negative control product, cross-contamination occurred during the PCR set-up and the test must be repeated.

2 The volume of $H_2O$ depends of the volume of DNA used in the reaction.

#### 1.6.3. PCR amplification and detection of Borghi and SG33 vaccine strains

i) To identify vaccine strains by PCR the following couple of primers are used:

VAX-F: 5'-ACA-AGA-ATA-TAC-TAA-AGA-ATA-CCA-CG-3' (138,997nt–139,021nt)

Borghi-R: 5'-TAG-CGC-GCA-TGG-CGA-CCC-TTG-GT-3' (139,398nt–139,420nt)

specific for Borghi vaccine strain (size of amplicon 405bp) and

VAX-F: 5'-ACA-AGA-ATA-TAC-TAA-AGA-ATA-CCA-CG-3' (138,997nt–139,021nt)

SG33-R: 5'-GAC-GTG-CAT-GGC-GAC-CCT-TTT-TGC-GTG-T-3'(139,398nt–139,419nt)

specific for SG33 vaccine strain (size of amplicon 409) (Cavadini *et al.,* 2010).

ii) The PCR amplification and analysis is set up as previously described with the difference of the annealing temperature of 56°C.

## 2. Serological tests

Infection of rabbits with MYXV strains induces a strong adaptive immunological response with the production of specific antibodies of the IgM and IgG classes (Kerr, 1997). This occurs also in the cases of vaccination with live vaccine or infection with low pathogenic MYXV strains, although antibodies titres in these cases are lower than those induced by high virulent strains. IgM appears 5–6 days post-infection and usually persists for 30–40 days whereas IgG peaks at 20–30 days remaining positive in naturally infected rabbits for at least 2 years. In relation to the value of titre of the dose, IgG antibodies to MYXV can be found in young rabbits up to approximately 2 months of age. As a consequence, MYXV serology is very useful for most of the purposes listed in Table 1. However it must be considered that protection of rabbits from myxomatosis is dependent more on the cell-mediated immune response than the serum antibodies. As consequence anti-MYXV antibodies titres are not a direct indication of the level of disease protection. Finally, considering the very limited degree of genetic variation of the immuno-dominant MYXV proteins (i.e. immunodominant envelope protein – IMV – M071L) serology cannot be used for typing different field isolates of MYXV.

Numerous methods have been used to detect serum anti-MYXV antibodies, from traditional agar gel immunodiffusion to the more recent enzyme-linked immunosorbent assays (ELISAs). To date, ELISAs are preferred, for their simplicity, speed, low cost and high sensitivity and specificity. The complement fixation test (CFT) is no longer recommended because of its poor sensitivity (Gelfi *et al.,* 1999).

### 2.1. Indirect enzyme-linked immonosorbent assay (I-ELISA)

Two very similar indirect ELISAs (I-ELISA), with the antigen directly coated to the solid phase, were developed and used for MVXY serology. The results of the I-ELISA-1 described by Kerr (1997) have been compared with those obtained in neutralisation assay, whereas those obtained in the I-ELISA-2 developed by Gelfi *et al.* (1999) have been compared with indirect fluorescent antibody test (IFT) and CFT. Both ELISAs showed similar performances, consistently better than those of other serological methods.

#### 2.1.1. I-ELISA-1 (Kerr, 1997)

i) Antigen preparation

a) The Lausanna (LU) strain, isolated in Brazil in 1949, is considered *de facto* as the international reference MYXV strain (ATCC code VR-115). Alternatively, considering the high degree of antigenic stability of the MYXV, a regional high virulent isolate can be used as MYXV laboratory reference. This virus should be adapted to grow *in vitro* to obtain stocks with high titres in 48–72 hours of incubation.

b) Virus stocks are cultured in RK-13 or SIRC cells grown in MEM supplemented with 10% calf serum, 200 units/ml penicillin and 100 µg/ml streptomycin. The foci-forming assay may be employed for the viral titration as described below. Virus stocks are aliquoted and stored frozen at –80°C.

c) Grow virions in RK-13 or SIRC cells in 180 $cm^2$ tissue-culture flasks using a multiplicity of infection (m.o.i.) of 0.02–0.05 (approximately 1 infectious virion each 20–50 cultured cells). Infect 6–12 flasks for each preparation leaving grow the virus until uniform CPE is observed.

d) Wash the cell monolayers twice with PBS, pH 7.2, scrape the cells from the flask and pellet by centrifugation (800 ***g*** for 10 minutes at 4°C).

e) Resuspend pellets in 5 ml cold PBS and sonicate to release the intracellular virus. Digest the suspension with DNase1 (25 µg/ml) and RNsaeA (50 µg/ml) at 37°C for 30 minutes with frequent agitation.

f) Pellet the virions by centrifugation (250,000 ***g***, 20 minutes at 4°C) throughout a step gradient formed by overlaying 10% of dextran T10 with the same volume of 36% of sucrose, both solutions in 10 mM Tris/HCl, pH 8.0, and 1 mM EDTA.

g) Resuspend the pellet in cold PBS in the original volume and repeat the above pelleting step. Then, resuspend the pellet in 0.5–1.0 ml cold PBS, aliquot and store frozen at –20°C as antigen stock.

h) To establish the dilution at which to use the antigen in the ELISA, titrate the stock antigen against a positive reference serum to produce an optical density (OD) of 1.0 at a serum dilution of 1/100. Perform this step using the ELISA protocol given below.

ii) Quantification of MYXV by foci-forming assay (Smallwood *et al.,* 2010)

a) Seed a six-well tissue culture plate with 1/5 of a nearly confluent 75 $cm^2$ tissue culture flask (approximately 2–4 × $10^5$ cells) of CV-1 cells (African green monkey kidney fibroblast) or alternatively RK-13 cells.

b) Mix the cells with complete MEM to a final volume of 12 ml for seeding each of the six wells. Pipette 2 ml into each well of the plate.

c) Incubate at 37°C in a $CO_2$ atmosphere. When cells appear to be 80–90% confluent (usually within 20–28 hours), proceed with the infection.

d) Before infection, check the viral preparation for the presence of clumps. If clumps are present or suspected, sonicate the solution with one single cycle of 10–15 seconds. During the sonication, keep the tube in ice.

e) Make serial ten-fold dilutions of virus in complete MEM starting from $10^{-2}$ to $10^{-8}$. Eliminate the media from the six-well plate and add 0.5 ml of each dilution to a single well.

f) Incubate the plate at 37°C a $CO_2$ atmosphere to obtain the adsorption of the virus to the cells. If a platform rocker is not available inside the incubator, every 10 minutes manually and gently rock the plate to redistribute the liquid.

g) After 1 hour add 1.5 ml of complete MEM to each well. Incubate 2–5 days at 37°C a $CO_2$ atmosphere.

h) Aspirate media from wells. Add 0.4–0.5 ml of crystal violet solution (0.1% [w/v] crystal violet dissolved in 20% ethanol) to each well, applying gently along the side of the wells to avoid removing cells.

i) Rock gently and incubate for up 1 hour at room temperature. Aspirate the staining and invert plates to dry the wells.

j) Under an inverted microscope, count foci in wells that have <100 (this should be possible for at least two consecutive wells).

k) Calculate the titre (FFU [foci-forming units]/ml): titre = number foci × dilution × 2.

iii) Production of standard laboratory reference sera

Use adult rabbits (2–3 months old) not vaccinated against myxomatosis and obtained from a farm or rabbitry free from myxomatosis.

a) Standard positive-control sera: vaccinate for myxomatosis 3–5 rabbits. After 30 days inoculate rabbits with a virulent strain of myxomatosis (preferably use the same strain used for antigen preparation). If the virus titre is known, inoculate 100–200 plaque-forming units (PFU) in 0.1 ml using the intradermal route. On day 30 after challenge, kill the rabbits, collect the blood and produce the sera following the standard protocol.

b) Standard negative-control sera: kill 3–5 rabbits, collect the blood and produce the sera following the standard protocol.

Store aliquoted sera at –20°C.

iv) ELISA protocol

Use high binding ELISA plates.

a) Coat the plate (50 µl in PBS, pH 7.2) with the antigen at the pre-determined dilution (see above) and incubate for 2 hours at 37°C.

b) Wash the plate with 5% (w/v) skim milk powder in PBS (milk/PBS). Block the plate with milk/PBS overnight at 4°C.

c) Distribute the sera on the plate

- Identification of negative and positive sera: Dilute 1/100 the standard and sample sera in milk PBS and add 50 µl per well. Leave two wells as blank (i.e. pipette 50 µl of milk/PBS only).
- Determination of the serum titre: All the sera are two-fold diluted starting from 1/100 dilution directly on the ELISA plate using a multichannel pipette. Leave two wells as blank (i.e. pipette 50 µl of milk/PBS only).

d) Incubate the plate for 2 hours at 37°C.

e) Wash three times with PBS and 0.05% Tween 20 (PBS/Tween).

f) Add an anti-rabbit Ig horseradish-peroxidase conjugate at the dilution suggests by the supplier in PBS/milk. Incubate for 30 minutes at 37°C.

g) Wash the plate six times using PBS/tween.

h) Add 100 µl of substrate (ABTS: 2,2-azino-bis 3-ethylbenzthiazoline sulphonate) at 1 mg/ml plus 0,06 % hydrogen peroxide, in 0.1 M citrate phosphate buffer at pH 4.0).

i) Incubate at room temperature and read the absorbance at 405 nm using an ELISA microplate reader spectrophotometer.

v) Interpretation of the results

Before analysing the ELISA results, subtract the mean OD value of the two blank wells from the OD value of all the sera included in the plate. After this, the OD value of the negative control serum should be lower than 0.1 OD.

a) A serum is classified as negative when the corresponding OD value is equal to or lower than the OD value of the negative control sera plus 0.1 OD.

b) A serum is classified as positive when the corresponding OD value is higher than the OD value of the negative serum plus 0.25 OD.

c) A serum with OD value above the negative cut off but below the positive cut off is considered equivocal or doubtful.

In case of end point titration of positive sera, the titre corresponds to the last dilution with an OD value still positive, that is an OD equal to the OD of the negative control sera at the dilution 1/100 (always subtracted by the background value) plus 0.1 OD.

### 2.1.2. I-ELISA-2 (Gelfi *et al.,* 1999)

i) Antigen preparation

This step in very similar to that of I-ELISA-1 but with the following minor differences.

a) RK-13 are grown in MEM medium supplement with 2% calf serum

b) Infected cells are scraped, pelleted by low-speed centrifugation and washed once in 20 mM Tris, 1 mM EDTA acid, 150 mM NaCl, pH 8.6 (TL20).

c) Cells are suspended in TL20, put on ice for 90 minutes (or overnight) and homogenised in a Dounce homogeniser.

d) After clarification of the homogenate at 1200 ***g*** for 10 minutes at 4°C, 7–9 ml of supernatant is layered on 2 ml of a 36% sucrose cushion in TL20 and ultracentrifuged at 200,000 ***g*** for 2 hours.

e) The pellet is resuspended in TL20 buffer at approximately 0.5 ml for each tube. Determinate protein concentration using a colorimetric methods (i.e. Bradford or BCA method).

ii) Production of standard laboratory reference sera

This step is identical to that described for I-ELISA-1

iii) I-ELISA method

Use high-binding ELISA plate.

a) Coat the plate using 1 µg/ml of antigen in PBS pH 7.6 and incubate overnight at 37°C.

b) Wash the plate three times with PBS and block the plate by incubation in 15 mg/ml gelatin in PBS for 1 hour at 37°C.

c) Wash the plate three times in 0.1% PBS/Tween 20. Sera are serially two-fold diluted in PBS/Tween starting from the dilution 1/50. Include among the sera the negative and positive controls. Leave two wells with only PBS in place of the sera. Incubate the plate for 1 hour at 37°C.

d) Wash the plate three times in PBS/Tween. Add a goat IgG anti-rabbit IgG conjugated to alkaline phosphatase diluted in PBS/Tween at the dilution indicated by the supplier. Incubate the plate for 1 hour at 37°C.

e) Wash the plate four times with PBS-Tween. Add disodium p-nitrophenyl phosphate at a concentration of 1 mg/ml in 10% diethanolamine. Keep the plate at room in the dark for 12 minutes and then stop the reaction adding 2 N NaOH. Read the absorbance values at 405 nm using an ELISA microplate reader spectrophotometer.

iv) Interpretation of the results

Use the OD value of the negative control sera as a reference in the interpretation of the results. This value should be equivalent to the average OD value of the wells containing PBS only. The results are expressed as negative or positive at a specific serum dilution. The serum sample titre is expressed as the inverse of the highest dilution for which the OD value is greater than three times the absorbance of the control negative serum. Serum samples are considered positive starting from 1/100.

#### 2.1.3. Comments and suggestions on the use of I-ELISAs

Although considerable detail of the methods has been given above, each laboratory should standardise and validate them in relation to local conditions, as well as taking into account the disease epidemiology in the area. In addition, the anti-Ig rabbit enzyme conjugate is one of the most critical reagents in terms of specificity and sensitivity of the reaction. Considering that enzyme-Ig conjugate supplied by different companies or from different batches can show marked differences in technical performance, a new partial standardisation is necessary in each case. It may also be possible to improve antigen production (i.e. higher titres and degree of purification) using well described methods (Smallwood *et al*., 2010).

Although not specifically included in the above methods, laboratory procedures could also include the following steps:

i) After the coating step, to store the plates at –20°C in plastic bag for at least 3 weeks.

ii) To limit or better avoid antigen freezing and thawing, to add 50% glycerol to the antigen preparation that is then stored at –20°C at a liquid state.

### 2.2. Competitive ELISA (MYXV-C-ELISA)

At the OIE Reference Laboratory for Myxomatosis, serological tests are routinely performed by using a competitive ELISA (C-ELISA), centred on the use of a MAb (1E5) that specifically recognises the MYXV immunodominant envelope protein (IMV – open reading frame M071L). The main characteristics of this C-ELISA are:

i) the detection of all the anti MYXV immunoglobulin classes present in the serum,

ii) higher specificity than I-ELISA,

iii) the specificity is further increased by the use of MAb 1E5 anti-IMV protein.

The OIE Reference Laboratory can supply the main reagents for the MYXV C-ELISA in a kit format that includes detailed instruction of the methods including the result interpretation.

### 2.3. Preparation of standard reagents for AGID and IFAT

#### 2.3.1. Preparation of antigen

Antigen is produced from cell cultures using the RK-13 cell line as described above (Section B.2.1.1.i). The monolayer is harvested about 48 hours after infection, when the cells clearly show CPE (80%), and is centrifuged (1000 ***g***). The supernatant fluid is retained. Infected cells are frozen and thawed three times to release additional virus and the viral suspension is clarified at 1000 ***g***. The newly harvested supernatant is added to the original supernatant. The final supernatant fluid is the antigen, and is stored at –20°C or –70°C (for longer conservation). It is titrated in cell cultures before use.

#### 2.3.2. Production of standard laboratory reference sera

This step is identical to that described for I-ELISAs (Section B.2.1)

### 2.4. Indirect fluorescent antibody test (IFAT)

IFAT (Gilbert *et al*., 1989) is carried out using RK-13 cell cultures in flat-bottomed wells of microtitre plates:

#### 2.4.1. Test procedure

i) Cell suspensions, $4 \times 10^4$ cells diluted in medium, are distributed in all wells and a confluent cell sheet is formed within 24 hours.

ii) The medium is discarded and 100 µl of viral suspension (with a multiplicity of infection of 0.05) is added to each well.

iii) After 2 hours, 100 µl MEM containing 2% calf serum is added.

iv) After 48 hours of incubation, the plates are washed with PBS and fixed with acetone containing 50% ethanol for 30 minutes at –20°C, or paraformaldehyde (4% in PBS) at room temperature.

v) The plates are then dried at 37°C for 15 minutes. The plates can be stored at –30°C or –70°C for 3 months.

vi) Sera are tested by IFA using anti-rabbit IgG conjugated to fluorescein isothiocyanate.

vii) The test results may be qualitative with sera diluted 1/20, or quantitative with serial dilutions of serum.

### 2.5. Agar gel immunodiffusion test

Agar is prepared as described previously (Section B.1.5.1) using 6 ml per 10 cm Petri dish.

#### 2.5.1. Test procedure

i) Strips of filter paper containing the standard antigen and antiserum, and discs containing test sera are arranged on the surface of the agar (discs between the strips).

ii) The plates are incubated in a humid atmosphere at 37°C and read after 24–48 hours.

iii) Three precipitation lines should appear. If the test sera contain MYXV-specific antibody, at least one of the three lines is bent towards the antigen band; otherwise it remains straight. If sera contain MYXV antigen, at least one of the lines is bent towards the standard serum strip. The test can also be carried out in a more conventional manner using liquid reagents in wells cut in the agar.

## C. REQUIREMENTS FOR VACCINES

## 1. Background

Two types of live vaccine have been developed for vaccination against myxomatosis: heterologous vaccines prepared from Shope fibroma virus (SFV) (Fenner & Woodroofe, 1954; Shope, 1932), and homologous vaccine prepared from attenuated strains of MYXV (Arguello Villares, 1986; Gorski & Mizak, 1985; Saurat *et al*., 1978; Tozzini & Mani, 1975; Von Der Ahe *et al*., 1981). They are both administered subcutaneously or intradermally.

Each type of vaccine has its advantages and disadvantages. SFV-based vaccines may be considered less immunogenic. Their use has greatly diminished in recent years and they are not generally used in the meat rabbit industry. Live attenuated MYXV vaccines are more immunogenic and the duration of protection is longer, i.e. around 4–6 months. However, they may be immunosuppressive, particularly in young rabbits (Fenner & Woodroofe, 1954; McKercher & Saito, 1964).

A recombinant attenuated live MYXV strain expressing rabbit haemorrhagic disease virus (RHDV) capsid protein and conferring double protection against myxomatosis and RHDV (Bertagnoli *et al*., 1996) has been developed and it is commercially available in Europe (Spibey *et al*., 2012). An attenuated field strain of MYXV from Spain (strain 6918) has been similarly engineered and tested in laboratory and field studies as a vaccine against both myxomatosis and RHDV for wild rabbits (Angulo & Barcena, 2007).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

## 2. Outline of production and minimum requirements for vaccines

### 2.1. Characteristics of the seed

#### 2.1.1. Biological characteristics of the master seed

A master seed virus (MSV) must be established and used according to a seed-lot system. A record must be kept of its origin, passage history and characteristics.

The viruses employed for vaccine are SFV or MYXV attenuated strains.

i) Shope fibroma virus heterologous vaccine

The strains of SFV are usually the original Shope's OA strain (1932), Boerlage's strain, IA variant or various closely related strains. Specific antigenic characteristics of the SFV strains are verified by AGID using monospecific sera against fibroma virus and MYXV.

SFV strains are maintained by passage in specific pathogen free (SPF) rabbits or in unvaccinated rabbits from a stock known to be free (serologically negative) from myxomatosis. Skin on the backs of healthy adult rabbits is shaved, and multiple sites are inoculated with a 1% suspension of virulent material. Fibromas are fully developed within 8–10 days, at which time the rabbits are killed and the tumours are removed aseptically and homogenised with distilled water. The suspension is stored at –30°C or –70°C in 50% buffered glycerol, or as a 5% dilution in a protein solution (bovine albumin). The production of the SFV is also possible in rabbit dermal cell line.

ii) Live attenuated homologous myxomatosis vaccine

The strains of MYXV are field strains attenuated by serial passaging in embryonated chicken eggs, rabbit kidney cells at decreasing temperatures, or chicken embryo cells. The strains usually result from having been cloned several times. Various attenuated strains have been obtained for commercial vaccine preparation (MSD, SG33, Borghi, BT 84, MAV, Leon 162, Poxlap, Pisa, etc.).

The identity of MYXV is confirmed by neutralisation test, FAT or IPMA in RK-13 cells or other suitable cell lines, using a monospecific antiserum (produced by vaccination of rabbits with the specific vaccine viral strain). Identity of MYXV can also be achieved by using molecular methods (i.e. PCR using specific primers). In this way it is also possible to better characterise the genomic properties of the attenuated viral strain.

MYXV may be grown on chicken embryo cell culture obtained from flocks free from specified pathogens. MYXV can also be cultivated on suitable cell lines (rabbit dermal cell line) and on RK-13 cells.

iii) Recombinant attenuated live MYXV strain expressing RHDV

The vaccine is constructed from a laboratory-attenuated strain of myxoma virus and the capsid protein gene of an RHDV isolate (Bertagnoli *et al.,* 1996; Spibey *et al.,* 2012). Standard laboratory methods for MYXV live attenuated vaccine are used and the RHDV

capsid gene was inserted into the MGF/M11L locus of the myxoma virus genome. Vaccine material is prepared in rabbit kidney cells (RK-13).

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

The master seed must be free from bacterial, fungal, mycoplasmal and viral contamination. Purity is determined by testing for a variety of contaminants, i.e. extraneous viruses, bacteria, mycoplasmas and fungi. Tests for sterility and freedom from contamination of biological materials should be performed according to chapter 1.1.4.

In particular, testing for contaminating viruses is done by inoculating a confluent monolayer of Vero cells. Vaccine, adjusted to the equivalent of 20 doses/ml, is neutralised with an equal volume of monospecific hyperimmune serum for 30 minutes at 37°C. The mixture is filtered through a 0.22 µm membrane filter, and 1 ml volumes are inoculated into five 25 ml bottles of cell cultures. These are kept under observation for 7 days. After harvesting, the cells are suspended in medium and subjected to several freeze–thaw cycles, followed by centrifugation and filtration, and the material is inoculated into fresh cultures and observed for 7 days. There should be no evidence of CPE or, for excluding the presence of RHDV, haemagglutination of human O RBCs.

#### 2.1.3. Validation as a vaccine strain

The test is carried out for each route of administration indicated. Use at least 10 rabbits, of the minimum age recommended for vaccination and negative for myxomatosis (serologically negative). Administer by a recommended route a quantity of virus corresponding to not less than 10 times the maximum titre that may be expected in a dose of vaccine. Observe the rabbits for 28 days. Record the body temperature the day before vaccination, at vaccination, 4 hours after vaccination and then daily for 4 days; note the maximum temperature increase for each animal. No abnormal local or systemic reaction occurs; the average temperature increase does not exceed 1°C and no animal shows a rise greater than 2°C. A local reaction lasting less than 28 days may occur.

The innocuity of the viral strains (both SFV and MYXV), used for vaccine preparation, for pregnant females and for suckling rabbits should be assessed (see Section C.2.3.2). They should be safe also for other species (e.g. guinea-pigs, adult mice and hares).

### 2.2. Method of manufacture

#### 2.2.1. Procedure

i) Shope fibroma virus heterologous vaccine

The original production of SFV was by multiple intradermal inoculations of seed virus into the skin on the back of a number of rabbits. The product of fibroma homogenate can be stored by freezing or used immediately. Production is nowadays possible in rabbit dermal cell line such as RK-13 (Jerabek, 1980). After clarification by centrifugation, the supernatant fluid is mixed with a stabiliser containing antibiotics and is distributed into ampoules or bottles for lyophilisation. Kaolin may be added as an adjuvant (40 mg/ml) to strengthen intensity and duration of immunisation, in which case the vaccine is administered subcutaneously.

ii) Live attenuated homologous myxomatosis vaccine and recombinant attenuated live MYXV+RHDV

MYXV is propagated in a suitable cell line (e.g. RK-13). Virus is harvested after 2–6 days. The viral suspension may be stored at –70°C. The vaccine is prepared by diluting in specified proportions the viral preparation with a stabiliser for lyophilisation. After homogenisation, the product is distributed into bottles for lyophilisation, the bottles being sealed under vacuum or in sterile nitrogen.

#### 2.2.2. In-process control

The batch potency is determined by measurement of virus content. Serial dilutions of the vaccine (SBV or MYXV) are inoculated into suitable cell cultures. One dose of vaccine shall contain not less than the minimum titre previously established. If the vaccine strain is not adapted to cultures, an efficacy test in rabbits shall be carried out (see Section C.2.3.3).

i) Shope fibroma virus heterologous vaccine

The SFV titre was originally measured *in vivo* by calculating the $ID_{50}$ after intradermal inoculation of serial dilutions of the clarified supernatant fluid into several sites (e.g. five) on up to six rabbits. A dilution of a standard preparation of SFV was also inoculated into each rabbit to confirm the animal's correct response to inoculation.

The titration can also be performed in RK-13 cells ($TCID_{50}$).

ii) Live attenuated homologous myxomatosis vaccine and recombinant attenuated live MYXV+RHDV

Titration of MYXV can be done in RK-13 cells ($TCID_{50}$).

In each case the titre should correlate with the required potency as defined by the test for batch potency, see Section C.2.3.3.

### 2.2.3. Final product batch tests

i) Sterility

See Section C.2.1.2.

ii) Identity

The identity of SFV and MYXV are determined in RK-13 cells by one of the methods of identification of the agent (e.g. FAT or IPMA using monospecific serum or MAbs).

iii) Safety

Samples for safety testing are taken from a batch produced according to the manufacturing process. The dose to be used shall contain the maximum titre or potency established by the manufacturer (release titre).

a) Shope fibroma virus heterologous vaccine

The pathogenicity of the SFV strains is tested by inoculating rabbits with serial dilutions of supernatant fluids obtained by centrifugation of tumour preparations. Macroscopic and histopathological features and the course of development of fibromas are tested in SPF rabbits periodically. To note that numerous serial passages in rabbits may induce mutation to the inflammatory IA strain, which produces severe lesions that are more inflammatory than neoplastic.

b) Live attenuated homologous myxomatosis vaccine and recombinant attenuated live MYXV+RHDV

The residual pathogenicity of the MYXV strains is tested by intradermal inoculation into SPF rabbits or unvaccinated rabbits free from myxomatosis (serologically negative). These rabbits should not develop more than a local reaction with possibly small secondary lesions on the head that disappear within a few days.

For all types of vaccines, the rectal temperature and the body weight should be recorded as additional parameters. Behavioural and feeding/drinking changes should be also monitored. Rabbits should be put under observation for 28 days.

## 2.3. Requirements for authorisation/registration/licensing

### 2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine (see Section C.2.2) and quality control testing (see Section C.2.1.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume. In-process controls are part of the manufacturing process.

### 2.3.2. Safety requirements

Several tests are performed to demonstrate different aspects of safety. The safety of ten times the normal dose must be demonstrated. Also, it is necessary to examine the organ dissemination of vaccine virus within the vaccinated animal, the ability of vaccine virus to spread

from the vaccinated animal to in-contact animals and to test whether there is reversion-to-virulence of the vaccine virus.

i) To test the use of ten times the normal dose

After rehydration, ten doses of the lyophilised SFV vaccine are injected subcutaneously into each of three susceptible rabbits, which are then observed for 21 days. Local reactions should be slight with no generalisation and effect on general health. MYXV vaccine is tested using ten doses injected intradermally into the ears of three susceptible rabbits, which are then observed for 21 days. The primary myxoma lesion should remain mild and no abnormal local or systemic reaction should occur,

ii) To test the use of the vaccine in pregnant rabbits

Administer the virus to not less than ten pregnant rabbits according to the schedule to be recommended on the label. Prolong the observation period until 1 day after parturition. The rabbits remain in good health and there is no abnormal local or systemic reaction. No adverse effects on the pregnancy or the offspring are noted.

iii) To test the potential increase in virulence (reversion-to-virulence)

Administer by a recommended route to each of two rabbits, 5–7 weeks old and that do not have antibodies against myxoma virus, a quantity of virus that will allow recovery of virus for the passages described below. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. Kill the rabbits 5–10 days after inoculation and remove from each rabbit organs, or tissues with sufficient virus to allow passage; homogenise the organs and tissues in a suitable buffer solution, centrifuge the suspension and use the supernatant for further passages. Inoculate the supernatant into suitable cell cultures to verify the presence of virus. Administer by an appropriate route, at a suitable rate, a suitable volume of the supernatant to each of two other rabbits of the same age and the same susceptibility. This operation is then repeated at least five times. If the virus has disappeared, a second series of passages is carried out. Inoculate virus from the highest recovered passage level to rabbits, observe for 28 days and compare any reactions that occur with those seen in the test for safety described above. There is no indication of an increase in virulence as compared with the non-passaged virus. If virus is not recovered in either of two series of passages, the vaccine virus also complies with the test.

### 2.3.3. Efficacy requirements

Different trials must be undertaken from representative batches of final product containing the minimum titre or potency. The same protocol is adopted for both SFV and MYXV vaccines. The protective effect is demonstrated as follows:

A minimum of ten adult rabbits is inoculated with a dose of vaccine, and five rabbits serve as unvaccinated controls. After not less than 21 days after vaccination, all rabbits are inoculated by a suitable route (e.g. intradermally into the eyelids or in two sites on the flank), with a pathogenic strain of MYXV (example: 0.1 ml inoculum of the Lausanne virus strain containing $10^3$ $ID_{50}$ [median infectious dose]). Observe the rabbits for a further 21 days. The test is not valid if less than 90 per cent of the control rabbits display typical signs of myxomatosis. A vaccine containing MYXV complies with the test if not less than 90 per cent of vaccinated rabbits show no signs of myxomatosis. A vaccine containing SFV complies with the test if not less than 75 per cent of vaccinated rabbits show no signs of myxomatosis.

The manufacturer shall have established a minimum titre or potency taking into account loss in potency during the shelf life.

### 2.3.4. Duration of immunity

Several groups of ten susceptible rabbits are vaccinated. One batch is tested by challenge infection (as in efficiency test, see C.2.3.3), at 1, 2, 3, etc., months post-vaccination for SFV, and at 1, 3, 6, and 9 months for MYXV. The duration of immunity is deduced from the time during which not less than 90 per cent of rabbits vaccinated with a vaccine containing MYXV or not less than 75 per cent of SFV vaccinated rabbits show no signs of myxomatosis.

### 2.3.5. Stability

Stability studies (based on an acceptable potency test) are required to establish the validity of the expiry date that appears on the product package. Some authorities allow the use of

accelerated stability tests to determine a provisional expiry date for products, e.g. incubating at 37°C for 1 week for each year of dating. Such estimates must be confirmed by periodic real-time potency tests on at least three different batches/serials through the period of time indicated by the expiry date, and 3–6 months beyond. For attenuated live vaccines, testing should be done at release and at the approximate expiry date until a statistically valid record has been established.

Titrations of vaccine virus are carried out at intervals until 3 months beyond the requested shelf life on at least three batches of vaccine.

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

**NB:** FIRST ADOPTED IN 1990; MOST RECENT UPDATES ADOPTED IN 2014.