CHAPTER 2.1.23.

VESICULAR STOMATITIS

SUMMARY

Vesicular stomatitis (*VS*) *is a vesicular disease of horses, cattle and pigs caused by vesiculoviruses of the family Rhabdoviridae. This disease is clinically indistinguishable in relevant susceptible species from foot and mouth disease* (*FMD*)*, vesicular exanthema of swine* (*VES*)*, or swine vesicular disease* (*SVD*)*. Sheep, goats and many other wild species can be infected. Humans are also susceptible. The disease is limited to the Americas; however, it was previously described in France and in South Africa.*

Virus is transmitted directly by the transcutaneous or transmucosal route and has been isolated from sandflies and mosquitoes. Experimental transmission has been shown from black flies to both pigs and cattle. There is seasonal variation in the occurrence of VS: it disappears at the end of the rainy season in tropical areas, and at the first frosts in temperate zones. The pathogenesis of the disease is unclear, and it has been observed that the specific circulating antibodies do not always prevent infection with VS serogroup viruses.

Although VS may be suspected when horses are involved as well as pigs and cattle, prompt differential diagnosis is essential because the clinical signs of VS are indistinguishable from FMD when cattle and pigs are affected, and from SVD or VES when only pigs are affected.

Identification of the agent: *Virus can be readily isolated by the inoculation of several tissue culture systems, or embryonated chicken eggs. Viral RNA can be detected from epithelial tissue and vesicular fluid by conventional and real-time reverse transcriptase polymerase chain reaction* (*RT-PCR*). *Viral antigen can be identified by an indirect sandwich enzyme-linked immunosorbent assay* (*IS-ELISA*) *– this is the least expensive and most rapid test. The complement fixation test* (*CFT*) *is also a good alternative. The virus neutralisation* (*VN*) *test may be used, but it is elaborate and time-consuming.*

Serological tests: *Convalescent animals develop serotype-specific antibodies within 4–8 days of infection that are demonstrated by a liquid-phase blocking ELISA* (*LP-ELISA*), *a competitive ELISA* (*C-ELISA*) *and VN. Other described tests are CFT, agar gel immunodiffusion and counter immunoelectrophoresis.*

Requirements for vaccines: *Inactivated virus vaccines with aluminium hydroxide or oil as adjuvants have been tested in the United States of America and in Colombia, respectively. Both vaccines generated high levels of specific antibodies in the sera of vaccinated cattle. However, it is not yet clear if serum antibodies would prevent the disease. An attenuated virus vaccine has been used in the field with unknown efficacy.*

A. INTRODUCTION

Vesicular stomatitis (VS) was described in the United States of America (USA) by Oltsky *et al*. (1926) and Cotton (1927) as a vesicular disease of horses, and subsequently of cattle and pigs. Vesicles are caused by virus on the tongue, lips, buccal mucosa, teats, and in the coronary band epithelium of cattle, horses, pigs, and many other species of domestic and wild animals. Natural disease in sheep and goats is rare, although both species can be experimentally infected. Mixed infections of foot and mouth disease (FMD) and VS viruses have occurred in the same herds of cattle and can be induced experimentally. Many species of laboratory animals are also susceptible. The disease is limited to the Americas; however, it was described in France (1915 and 1917) and in South Africa (1886 and 1897) (Hanson, 1952).

Influenza-like signs, normally without vesicles, have been observed in humans who are in contact with animals with VS or who handle infective virus. All manipulations involving virus, including infective materials from animals, should be undertaken with using proper biosafety procedures.

Two distinct immunological serotypes of vesicular stomatitis virus (VSV) are recognised: New Jersey (NJ) and Indiana (IND). Both viruses are members of the genus *Vesiculovirus*, family Rhabdoviridae and have been extensively studied at the molecular level. Several other closely related rhabdoviruses have been isolated from sick animals over the past decades. There are three subtypes of the IND serotype based on serological relationships: IND-1 IND-2 and IND-3; they are also known as classical IND virus (VSIV), cocal virus (COCV), and alagoas virus (VSAV), respectively (Federer *et al*., 1967). Strains of the serotype NJ and subtype IND-1 are endemic in livestock in areas of southern Mexico, Central America, Venezuela, Colombia, Ecuador and Peru, with VSV NJ causing the vast majority (>80%) of the clinical cases. Sporadic activity of NJ and IND-1 VSV has been reported in northern Mexico and the western United States. IND-2 has only been isolated in Argentina and Brazil and only from horses (Salto-Argentina/63, Maipú-Argentina/86, Rancharia-Brazil/66, Riberao-Brazil/79) (Alonso *et al*., 1991; Alonso Fernandez & Sondahl, 1985). Cattle living together with the affected horses did not develop antibodies against VSV (Alonso *et al*., 1991). The IND-3 subtype, (Alagoas-Brazil/64), has been identified, sporadically only in Brazil and only in horses until 1977. However, in 1977 the IND-3 subtype (Espinosa-Brazil/77 strain) was first isolated from cattle in Brazil; it has been observed that this subtype affects cattle to a lesser degree than horses (Alonso *et al*., 1991; Alonso Fernandez & Sondahl, 1985). This finding confirms the first descriptions, in 1926 and 1927 (Cotton, 1927; Oltsky *et al*., 1926), of the NJ and IND serotypes in horses, and subsequently in cattle and pigs; this same predilection has been observed in other VS outbreaks.

The mechanism of transmission of the virus is unclear. The viruses have been isolated from sandflies, mosquitoes, and other insects (Comer *et al*., 1992; Francy *et al*., 1988; Mason, 1978). Experimental transmission of VS NJ has been demonstrated to occur from black flies (*Simulium vittatum*) to domestic swine and cattle (Mead *et al*., 2004; 2009). During the 1982 epizootic in the western USA, there were a number of cases where there was direct transmission from animal to animal (Sellers & Maarouf, 1990). VSV has historically been considered to be endemic in feral pigs on Ossabaw Island, Georgia, USA (Boring & Smith, 1962), but recent evidence suggests it may have disappeared from the island (Killmaster *et al*., 2011).

The incidence of disease can vary widely among affected herds. Usually 10–15% of the animals show clinical signs. Clinical cases are mainly seen in adult animals. Cattle and horses under 1 year of age are rarely affected. Mortality is close to zero in both species. However, high mortality rates in pigs affected by the NJ virus have been observed. Sick animals recover in about 2 weeks. The most common complications of economic importance are mastitis and loss of production in dairy herds (Lauerman *et al*., 1962). Recent VSV outbreaks in the USA have been associated primarily with horses and the NJ serotype.

B. DIAGNOSTIC TECHNIQUES

VS cannot reliably be clinically differentiated from the other vesicular diseases in the relevant susceptible species, such as FMD, vesicular exanthema of swine (VES), and swine vesicular disease (SVD). An early laboratory diagnosis of any suspected VS case is therefore a matter of urgency.

The sample collection and technology used for the diagnosis of VS must be in concordance with the methodology used for the diagnosis of FMD (chapter 2.1.8), VES and SVD, in order to facilitate the differential diagnosis of these vesicular diseases. Note: VS viruses can be human pathogens and appropriate precautions should be taken when working with potentially infected tissues or virus (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

Vesicle fluid, epithelium covering unruptured vesicles, epithelial flaps of freshly ruptured vesicles, or swabs of the ruptured vesicles are the best diagnostic samples. These samples can be collected from mouth lesions, as well as from the feet and any other sites of vesicle development. It is recommended that animals should be sedated before samples are collected to avoid injury to helpers and for reasons of animal welfare. Samples from all species should be placed in containers of Tris-buffered tryptose broth with phenol red, pH 7.6. If complement fixation (CF) is to be carried out for antigen detection, samples from all species can be collected in glycerol/phosphate buffer, pH 7.2–7.6. (Note: glycerol is toxic to cell cultures and decreases the sensitivity of virus isolation; it is therefore only recommended for collection of samples for CFT.) Samples should be sent to the laboratory on ice packs if they can arrive at the laboratory within 48 hours after collection. If samples require more than 48 hours transit time they should be sent frozen on dry ice with precautions to protect the sample from direct contact with CO_2. There are special packaging requirements for shipping samples with dry ice (see Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens* for further information on shipping of diagnostic samples). Alternatively, samples can be shipped with commercially available freezer packs that have been frozen in an ultralow freezer (–60°C or colder) if shipping time is of short duration.

When epithelial tissue is not available from cattle, samples of oesophageal–pharyngeal (OP) fluid can be collected by means of a probang (sputum) cup. In pigs, throat swabs can be taken for submission to a laboratory

for virus isolation. This material should be sent to the laboratory refrigerated in Tris-buffered tryptose broth. If the samples will be in shipment for more than 48 hours after collection, they should be sent frozen with dry ice as described previously. Probang samples for isolation of virus should not be treated with solvents such as chloroform. Virus can be isolated from oral and nasal specimens up to 7 days post-infection.

When it is not possible to collect samples for identification of the agent, serum samples can be used for detecting and quantifying specific antibodies. Paired sera from the same animals, collected 1–2 weeks apart, may be needed depending on the serological assay being used and prior history of VSV in the country.

Specific reagents for VS diagnosis are not commercially available and each laboratory must produce its own or obtain them from a Reference Laboratory. The two OIE Reference Laboratories for vesicular stomatitis (see Table given in Part 4 of this *Terrestrial Manual*), and the Pirbright Institute, UK[1], produce and distribute diagnostic reagents on request.

Table 1. *Test methods available for the diagnosis of vesicular stomatitis and their purpose*

Method	Purpose					
	Population freedom from virus circulation	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[2]						
Virus isolation*	–	+	–	+++	–	–
IS-ELISA*	–	+	–	+++	–	–
CFT*	–	+	–	++	–	–
RT-PCR*	–	+	–	++	–	–
Detection of immune response[3]						
LP-ELISA**	++	+++	+++	+++	++	++
C-ELISA**	+++	+++	++	–	+++	++
VN**	++	+++	+++	+++	++	+++
CFT**	–	+	+	+++	+	–

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.
IS-ELISA = indirect sandwich enzyme-linked immunosorbent assay; CFT = complement fixation test; RT-PCR = reverse transcriptase polymerase chain reaction; LP-ELISA = liquid-phase blocking ELISA; C-ELISA = competitive ELISA; VN = virus neutralisation
*Should only be used on animals demonstrating clinical signs compatible with VSV. A positive result is meaningful. A negative result could mean the animal is no longer shedding virus, the virus level is too low to detect, or, for virus isolation samples that the samples were not maintained at appropriate temperatures and received in an appropriate time period following collection for virus isolation (virus inactivated). **The presence of VSV antibodies only indicates prior exposure to VSV. It does not determine whether the antibodies are due to current infection or past infection. Interpretation of results needs to be based on serological results, clinical presentation, and epidemiology. CF antibody duration in an animal is generally less than 1 year. Antibodies detected by the VN assay and competitive ELISAs can be detected for years following infection. The difference in sensitivity of the serological assays has an effect on detection during the acute phase of infection; combination testing, such as C-ELISA and CF or paired sampling showing four-fold titre change (CF, VN, LP-ELISA), is therefore necessary when an animal presents with acute clinical signs of VSV. ***Indicates the presence of antibodies only; does not indicate protection from infection.

1 Pirbright Institute, Ash Road, Pirbright, Woking, Surrey GU24 0NF, United Kingdom.
2 A combination of agent identification methods applied on the same clinical sample is recommended.
3 One of the listed serological tests is sufficient.

1. Identification of the agent

1.1. Direct visualisation

Due to the different morphological characteristics of the rhabdovirus (VS serogroup viruses), picornavirus (FMD virus and SVD virus), calicivirus (VES) and the large number of virus particles present in vesicular fluids and epithelial tissues, electron microscopy can be a useful diagnostic tool for differentiating the virus family involved.

1.2. *In-vitro* cultivation

For identification of VS viruses and the differential diagnosis of vesicular diseases, clarified suspensions of field samples suspected to contain virus should be submitted for testing. For virus isolation, the samples are inoculated into appropriate cell cultures. The inoculation of African green monkey kidney (Vero), baby hamster kidney (BHK-21) and IB-RS-2 cell cultures with the same sample permits differentiation of the vesicular diseases: VS viruses cause a cytopathic effect (CPE) in all three cell lines; FMD virus causes a CPE in BHK-21 and in IB-RS-2, while SVD virus causes a CPE in IB-RS-2 only. Many other cell lines, as well as most primary cell cultures of animal origin, are susceptible to VS serogroup viruses.

If a CPE develops in the cultures, the preferred immunological methods for the identification of the viral antigens in the laboratory are the ELISA (Alonso *et al*., 1991; Ferris & Donaldson, 1988), the CFT (Alonso *et al*., 1991; Jenny *et al*., 1958) and fluorescent antibody staining. The virus neutralisation (VN) test, with known positive antisera against the VS virus NJ and IND serotypes, may be used in tissue cultures or embryonated eggs, but it is more time-consuming.

1.3. *In-vivo* testing

Virus replicates and can be isolated in 8- to 10-day-old chicken embryos by inoculation into the allantoic sac.

1.4. Virus isolation

1.4.1. Test procedure

i) Inoculate cell culture in Leighton tubes and 25 cm^2 flasks with the clarified suspension of tissues or vesicular fluid.

ii) Incubate inoculated cell cultures at 37°C for 1 hour.

iii) Discard inoculum and wash cell cultures three times with cell culture medium and replace with cell culture medium containing 2.5% fetal bovine serum (FBS).

Iv) Incubate Leighton tube cell cultures at 33–35°C and observe for CPE.

v) After 18–24 hours of incubation, the cover-slip from one Leighton tube culture per specimen inoculated is stained with New Jersey and Indiana VS virus-specific fluorescent antibody (FA) conjugate.

vi) Remaining Leighton tube cultures and 25 cm^2 flask cultures are incubated at 35–37°C for 6 more days and observed daily for CPE.

vii) At 7 days post-inoculation, the remaining Leighton tube cover-slips are stained with FA conjugate.

viii) If CPE is observed and the FA staining is negative, a second passage is made, as described above, using the cells from the 25 cm^2 flask. Note: First passage cultures with significant CPE may yield false-negative immunofluorescence results. Serial tenfold dilutions may be prepared and inoculated to provide distinct plaques of fluorescing cells.

ix) *Interpretation of the results:* If no fluorescence is observed and no CPE evident in the flask culture, the sample is negative for virus isolation. If specific fluorescence is observed, the sample is positive for virus isolation.

x) Alternatively cell culture in flasks can be inoculated with field samples, incubated at 35–37°C for 48 hours and observed daily for CPE. If no CPE is observed after 48 hours, the flask cultures are frozen and thawed and a sample of the supernatant is inoculated into

fresh cell culture. Up to three passages are made, of 48 hours each. To detect the presence of VSV antigen, clarified supernatants of each passage are tested by ELISA, CFT or PCR.

1.5. Enzyme-linked immunosorbent assay

The indirect sandwich ELISA (IS-ELISA) (Alonso *et al*., 1991; Ferris & Donaldson, 1988) is currently the diagnostic method of choice for identification of viral serotypes of VS and other vesicular diseases. Specifically, the ELISA procedure with a set of polyvalent rabbit/guinea-pig antisera, prepared against virions of the representative strains of the three subtypes of the IND serotype, identifies all strains of the VS virus IND serotype (Alonso *et al*., 1991). For detection of VS virus NJ serotype, a monovalent set of rabbit/guinea-pig antisera is suitable (Alonso *et al*., 1991; Ferris & Donaldson, 1988).

1.5.1. Test procedure

i) *Solid phase:* ELISA plates are coated either for 1 hour at 37°C or overnight at 4°C with rabbit antisera and normal rabbit serum (as described in Alonso *et al*., 1991), and optimally diluted in carbonate/bicarbonate buffer, pH 9.6. Subsequently, the plates are washed once with phosphate-buffered saline (PBS) and blocked for 1 hour at room temperature with 1% ovalbumin Grade V (grade of purification) in PBS. After washing the plates can be used immediately or stored at –20°C for future use.

ii) *Test samples:* Antigen suspensions of test samples (10–20% epithelial tissue suspension, or musculo-skeletal tissue of chicken embryo in PBS or minimal essential medium (MEM) or undiluted clarified cell culture supernatant fluid) are deposited in the corresponding wells and the plates are incubated for 1 hour at 37°C on an orbital shaker.

iii) *Detector:* Monovalent and polyvalent guinea-pig antisera to VS virus NJ and IND serotypes, respectively, that are homologous to coated rabbit serum and that have been diluted appropriately in PBS containing 0.05% Tween 20, 1% ovalbumin Grade II, 2% normal rabbit serum, and 2% normal bovine serum (PBSTB) are added to the corresponding wells and left to react for 30–60 minutes at 37°C on an orbital shaker.

iv) *Conjugate:* Peroxidase/rabbit or goat IgG anti-guinea-pig Ig conjugate, diluted in PBSTB, is added and left to react for 30–60 minutes at 37°C on an orbital shaker.

v) *Substrate:* H_2O_2-activated substrate is added and left to react at room temperature for 15 minutes, followed by the addition of sulphuric acid to stop the reaction. Absorbance values are measured using an ELISA reader.

Throughout the test, 50 µl reagent volumes are used. The plates are washed three–five times between each stage with physiological saline solution or PBS containing 0.05% Tween 20. Controls for the reagents used are included.

vi) *Interpretation of the results:* Absorbance values of positive and negative antigen control wells should be within specified values for acceptance. Sample wells giving an absorbance ≥0.3 are considered to be positive for the corresponding virus subtype. Absorbance values <0.3–0.2 are considered suspicious and values <0.2 are considered negative for the corresponding virus subtype. Suspicious and negative samples should be inoculated in cell culture and passages re-tested in ELISA.

1.6. Complement fixation test

The ELISA is preferable to the CFT because it is more sensitive and it is not affected by pro- or anti-complementary factors. When ELISA reagents are not available, however, the CFT may be performed. The CFT in U-bottomed microtitre plates, using the reagents titrated by CF50% test, is described.

1.6.1. Test procedure

i) *Antisera:* Guinea-pig monovalent anti-NJ VS virus and polyvalent anti-IND VS virus, diluted in veronal buffer (VB) at a dilution containing 2.5 CFU_{50} (50% complement fixation units) against homologous virus, are deposited in plate wells. Those antisera are the detectors used in ELISA.

ii) *Test samples:* The antigen suspension of test samples, prepared as described for IS-ELISA, is added to the wells with serum.

iii) *Complement:* 4 CHU_{50} (50% complement haemolytic units) are added to the serum and antigen. (An alternative is to use 7.5, 10 and 20 CHU_{50} with the goal of reaching 4 CHU_{50}

in the test.) The mixture of antisera, test samples and complement is incubated at 37°C for 60 minutes.

iv) *Haemolytic system:* A suspension of sheep red blood cells (SRBC) in VB, sensitised with 10 HU_{50} (50% haemolytic units) of rabbit anti-SRBC serum, is added to the wells. The haemolytic system has an absorbance of 0.66 read at 545 nm, in the proportion of two volumes of haemolytic system + three volumes of distilled water. The mixture is incubated for 30 minutes at 37°C. Subsequently, the plates are centrifuged and the reaction is observed visually.

Volumes of 25 µl for antisera, test samples and complement, and 50 µl of haemolytic system, are required. Appropriate controls for the antisera, antigens, complement and haemolytic system are included.

It is possible to perform the CF50% test in tubes (Alonso *et al*., 1991) using reagent volumes of 200 µl (eight times greater than those indicated for the CF in microtitre plates). With the CF50% test, the reaction can be expressed as absorbance read spectrophotometrically at 545 nm.

v) *Interpretation of the results:* When controls react as expected, samples with haemolysis <20% for one antiserum in comparison with the other antiserum and controls are considered to be positive for the corresponding type.

Field samples that are negative on the ELISA or CFT should be inoculated into cell culture. If there is no evidence of viral infection after three passages, the specimen is considered to be negative for virus.

1.7. Nucleic acid recognition methods

The RT-PCR can be used to amplify small genomic areas of the VS virus (Hofner *et al*., 1994; Hole *et al.,* 2010; Rodriguez *et al*., 1993; Wilson *et al*., 2009). This technique will detect the presence of virus RNA in tissue and vesicular fluid samples and cell culture, but cannot determine if the virus is infectious. In general, PCR techniques have not been routinely used for screening diagnostic cases for viruses causing VS.

2. Serological tests

For the identification and quantification of specific antibodies in serum, the ELISA and the VN test are preferable. The CFT may be used for quantification of early antibodies. Antibody can usually be detected between 5 and 8 days post-infection; the length of time antibody persists has not been accurately determined for the three tests but is thought to be relatively short for the CF and for extended periods for the VN and ELISA (Katz *et al*., 1997).

2.1. Liquid phase blocking enzyme-linked immunosorbent assay

The liquid-phase blocking ELISA (LP-ELISA) is a method for the detection and quantification of antibodies to VS serogroup viruses. The use of viral glycoproteins as antigen is recommended because they are not infectious, allow the detection of neutralising antibodies, and give fewer false-positive results than the VN (Allende *et al*., 1992).

2.1.1. Test procedure

i) *Solid phase:* As described above in Section B.1.5 for the IS-ELISA.

ii) *Liquid phase:* Duplicate, two- to five-fold dilution series of each test serum, starting at 1/4, are prepared in U-bottomed microtitre plates. An equal volume of VS virus NJ or IND glycoprotein, in a predetermined dilution, is added to each well and the plates are incubated for 1 hour at 37°C. 50 µl of these mixtures is then transferred to the ELISA plates with the solid phase and left to react for 30 minutes at 37°C on an orbital shaker.

iii) *Detector, conjugate and substrate:* The same steps described for the IS-ELISA are performed using monovalent antisera homologous to the test antigen, as detectors

iv) *Interpretation of the results:* 50% end-point titres are expressed in $\log_{10}$ in reference to the 50% OD of the antigen control, according to the Spearmann–Kärber method. Titres of >1.0 (1/10) are considered to be positive.

2.2. Competitive enzyme-linked immunosorbent assay

A competitive ELISA for detection of antibodies has also been developed. The procedure described here is based on a procedure described by Afshar *et al.* (1993). It uses vesicular stomatitis NJ and IND-1 recombinant antigens as described by Katz *et al.* (1995).

2.2.1. Test procedure

i) *Solid phase:* Antigens are diluted in carbonate/bicarbonate buffer, pH 9.6, and 75 µl is added to each well of a 96-well ELISA plate. The plates are incubated overnight at 4°C; coated plates can be frozen, with antigen *in situ*, at –70°C for up to 30 days. The plates are thawed, antigen is decanted, and 100 µl of blocking solution (5% nonfat dry milk powder solution in PBS [for example, 5 g dry milk powder dissolved in 95 ml PBS) is added. The plates are then incubated at 25°C for 15–30 minutes and blocking solution is decanted. The plates are washed three times with PBS/0.05% Tween 20 solution.

ii) *Liquid phase:* 50 µl of serum diluted 1/8 in 1% nonfat dry milk in PBS is added to each of the duplicate wells for each sample. A positive and negative control serum for each serotype should be included on each ELISA plate. The plates are incubated at 37°C for 30 minutes. Without washing, 50 µl of bioreactor fluid is added to each well and plates are incubated at 37°C for 30 minutes.

iii) *Detector:* The plates are washed three times, and 50 µl of goat anti-mouse horseradish-peroxidase conjugate diluted in 1% nonfat dry milk with 10% normal goat serum is added to each well. The plates are incubated at 37°C for 30 minutes, washed three times, and 50 µl of tetramethyl-benzidine (TMB) substrate solution is added to each well. The plates are incubated at 25°C for 5–10 minutes and then 50 µl of 0.05 M sulphuric acid is added to each well. The plates are read at 450 nm and the optical density of the diluent control wells must be > 1.0.

iv) *Interpretation of the results:* A sample is positive if the absorbance is ≤50% of the absorbance of the diluent control. Note that horses naturally infected with New Jersey virus have been known to test positive by this assay for at least 8years following infection.

2.3. Virus neutralisation

The VN test is carried out in tissue culture microtitre plates with flat-bottomed wells using inactivated serum as test sample, 1000 $TCID_{50}$ (50% tissue culture infective dose) of VS NJ or IND virus, and Vero M cells, or preformed monolayer (Allende *et al.,* 1992) or a suspension IB-RS-2 cells to test for the presence of unneutralised virus.

2.3.1. Test procedure

i) *Virus:* VS NJ or IND virus is grown in Vero cell monolayers and stored in liquid nitrogen or frozen at –70°C.

ii) *Test samples:* Sera are inactivated at 56°C for 30 minutes before testing. Positive and negative control standard sera are included in the test.

iii) *Virus neutralisation:* Sera are diluted in a two-fold or four-fold dilution series across the plates, starting from 1/4 dilution. Two rows of wells are used per serum. The same volume of NJ or IND VS virus suspension containing about 1000 $TCID_{50}$/25 µl is added and incubated at 37°C for 60 minutes to allow neutralisation to take place. Subsequently, 50 µl of the mixtures is deposited on preformed cell monolayers in microtitre plates or 150 µl of 300,000/ml IB-RS-2 or Vero cell suspension is added to each well with the serum/virus mixtures. The plates are covered with loosely fitting lids and incubated for 48–72 hours at 37°C in an atmosphere of 5% CO_2 or sealed with pressure-sensitive tape and incubated in a normal atmosphere. (It has been determined that a virus titre of 1000 $TCID_{50}$/25 µl will decrease the nonspecific reactions and maintain a high test sensitivity.)

iv) *Interpretation of the results:* Wells without CPE are considered to be positive. End-point titres of test serum titres are determined by the Spearmann–Kärber method when the virus titres are between 750 and 1330 $TCID_{50}$ and when titres of positive and negative standard sera are within twofold of their mean values as estimated from previous titration. The 100% neutralisation titres of each serum are expressed at log 10. Sera with values of 1/32 or greater are considered to be positive for antibodies against VSV. Note that horses naturally infected with New Jersey virus have been known to test positive by this test method for at least 8 years following infection. In an alternative protocol a viral dose of

1000 $TCID_{50}$ per millilitre of virus/serum mixture is used and reaction incubated at 37°C for 60 minutes to allow neutralisation to take place. Subsequently, 100 µl of the mixtures is deposited on preformed cell monolayers in microtitre plates. The plates are covered with loosely fitting lids and incubated for 48 hours at 37°C in an atmosphere of 5% CO_2. Wells without CPE are considered to be positive. End-point titres of test serum are determined by the Spearmann–Kärber method when the virus titres are between $10^{2\pm0.5}$/100 µl and when titres of positive and negative standard sera are within twofold of their mean values as estimated from previous titration. The 50% neutralisation titre of each serum is expressed as log 10. Sera with values of 1.3 (1/20) or greater are considered to be positive for VS antibodies (Allende *et al*., 1992).

The OIE Reference Laboratories for VS are currently evaluating and comparing the protocols described above. Both are widely used and can give acceptable results.

2.4. Complement fixation test

A detailed description of this test is given in Section B.1.6. This is modified as follows. The CFT may be used for quantification of early antibodies, mostly IgM. For this purpose, twofold serum dilutions are mixed with 2 CFU_{50} of known antigen and with 5% normal bovine or calf sera included in 4 CHU_{50} of complement. The mixture is incubated for 3 hours at 37°C or overnight at 4°C. Subsequently, the haemolytic system is added followed by incubation for 30 minutes at 37°C. The serum titre is the highest dilution in which no haemolysis is observed. Titres of 1/5 or greater are considered to be positive. This CF has low sensitivity and is frequently affected by anticomplementary or nonspecific factors.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Vesicular stomatitis virus infections can have significant impacts on the health and production aspects of animals, resulting in considerable economic losses for producers. Reduced feed intake caused by oral lesions can result in weight loss and delays to market. Lesions on the feet can cause temporary locomotor problems affecting the ability of an animal to obtain food and water, and permanent foot problems that result in the animal being culled. Lesions of the mammary gland can impact the ability of the dam to nurse her offspring and for harvesting milk for sale. Animals may be culled if mammary or teat lesions are severe. Where vaccination is practised, vaccine is used to reduce the severity of clinical signs and the economic impacts of the disease.

Attenuated virus vaccines have been tested in the field in the USA, Panama, Guatemala, Peru and Venezuela (Lauerman *et al*., 1962; Mason, 1978) with unknown efficacy. Killed vaccines for the Indiana and New Jersey serotypes are manufactured in Colombia and Venezuela (2002 OIE vaccine survey). Although a commercial vaccine combining VS and FMD antigens in a single emulsion for Andean countries has been tested in vaccination–challenge experimentation and published (House *et al*., 2003), the vaccine is not produced/applied routinely.

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 conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

Identity of the seed and the source of the serum used in growth and passage of the virus should be well documented, including the source and passage history of the organism.

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

The purity of the seed and cells to be used for vaccine production must be demonstrated. The master seed virus (MSV) should be free from adventitious agents, bacteria, or *Mycoplasma*,

using tests known to be sensitive for detection of these microorganisms. The test aliquot should be representative of a titre adequate for vaccine production, but not such a high titre that hyperimmune antisera are unable to neutralise seed virus during purity testing. Seed virus is neutralised with monospecific antiserum or monoclonal antibody against the seed virus and the virus/antibody mixture is cultured on several types of cell line monolayers. A cell line highly permissive for bovine viral diarrhoea virus, types 1 and 2, is recommended as one of the cell lines chosen for evaluation of the MSV. Bovine viral diarrhoea virus is a potential contaminant introduced through the use of fetal bovine serum in cell culture systems. Cultures are subpassaged at 7-day intervals for a total of at least 14 days, then tested for adventitious viruses that may have infected the cells or seed during previous passages.

2.2. Method of manufacture

2.2.1. Procedure

Once the vaccine is shown to be efficacious, and the proposed conditions for production are acceptable to regulatory authorities, approval may be granted to manufacture vaccine. Virus seed can be grown in cell culture. Selection of a cell type for culture is dependent on the degree of virus adaptation, growth in medium, and viral yield in the specific culture system. Vaccine products should be limited to the number of passages from the MSV that can be demonstrated to be effective. Generally, large-scale monolayer or suspension cell systems are operated under strict temperature-controlled, aseptic conditions and defined production methods, to assure lot-to-lot consistency. Dose of virus used to inoculate cell culture should be kept to a minimum to reduce the potential for viral defective interfering particles. When the virus has reached its appropriate titre, as determined by CPE, fluorescent antibody assay, or other approved technique, the virus is clarified, filtered, and inactivated (for killed vaccines).

2.2.2. Requirements for substrates and media

Cell cultures should be demonstrated free of adventitious viruses. All animal origin products used in the production and maintenance of cells (i.e. trypsin, fetal bovine sera) and growth of virus should be free of adventitious agents, with special attention paid to the presence of bovine viral diarrhoea virus.

2.2.3. In-process controls

Cell cultures should be checked macroscopically for abnormalities or signs of contamination and discarded if unsatisfactory. Virus concentration can be assessed using antigenic mass or infectivity assays.

An inactivation kinetics study should be conducted using the approved inactivating agent (β-propiolactone or ethylene-imine in binary form) on each viral lot with a titre greater than the maximum production titre and grown using the approved production method. This study should demonstrate that the inactivation method is adequate to assure complete inactivation of virus. Samples taken at regular timed intervals during inactivation, then inoculated on to a susceptible cell line, should indicate a linear and complete loss of titre by the end of the inactivation process.

During production, antigen content is measured to establish that minimum bulk titres have been achieved. Antigen content is generally measured before inactivation (if killed vaccine) and prior to further processing.

2.2.4. Final product batch tests

Vaccine candidates should be shown to be pure, safe, potent, and efficacious.

i) Sterility and purity

During production, tests for bacteria, *Mycoplasma*, and fungal contamination should be conducted on both inactivated and live vaccine harvest lots and confirmed on the completed product (see Chapter 1.1.9 *Tests of biological materials for sterility and freedom from contamination*).

ii) Safety

Final container samples of completed product from inactivated vaccines should be tested.

iii) Batch potency

Potency is examined on the final formulated product. Mirroring what is done for the potency test in foot and mouth disease vaccines, a vaccination–challenge test has being proposed for testing VSV vaccines (House *et al.,* 2003). The gaps in knowledge regarding the pathogenesis of VSV infection and the immune mechanism that affords protection against viral infection are limitations for development and implementation of a validated protocol for a challenge test. However, for batch release, indirect tests can also be used for practicability and animal welfare considerations, as long as correlation has been validated to protection in the target animal during efficacy tests. Frequently indirect potency tests include antibody titration after vaccination of target species. Ideally, indirect tests are carried out for each strain for one species and each formulation of vaccine to establish correlation between the indirect test results and the vaccine efficacy test results.

Relative potency could be used to determine antigen content in final product. It is necessary to confirm the sensitivity, specificity, reproducibility, and ruggedness of such assays.

2.3. Requirements for authorisation

2.3.1. Safety requirements

i) Target and non-target animal safety

Final product may be evaluated in the host animal using two animals of the minimum age recommended for use, according to the instructions given on the label; the animals are observed for 21 days. Field safety studies conducted on vaccinates, in at least three divergent geographical areas, with at least 300 animals per area, are also recommended.

For killed and modified live virus (MLV) vaccines product safety will be based on an absence of adverse reactions such as shock, abscesses at site of inoculation, etc. In the specific case of MLV vaccines, it would not be expected to see clinical signs. If clinical signs of vesicular stomatitis virus are observed, use of the vaccine should be reconsidered. Residual virus should be evaluated for prior to mixing the antigen with adjuvant. Initial safety is evaluated in a few animals for 21 days under close observation to assess for gross safety issues. If the vaccine passes this first safety test, the vaccine is used in the field in a larger number of animals to evaluate if subtle safety issues are present: adverse reactions/swelling, abscesses, shock, etc.

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

Reversion to virulence for live viral vaccines is often demonstrated by back passage through susceptible species. Virus is isolated from the vaccinated animal and the isolated virus is then used to inoculate additional animals. Sequential passage through animals should show that animals remain clinically healthy with no demonstration of typical vesicular stomatitis lesions.

iii) Environmental consideration

Inactivated vesicular stomatitis vaccines probably present no special danger to the user, although accidental inoculation may result in an adverse reaction caused by the adjuvant and secondary components of the vaccine. Modified live virus vaccines may pose a hazard to the user depending on the level of inactivation of the virus.

Preservatives should be avoided if possible, and where not possible, should be limited to the lowest concentration possible. Vaccine bottles, syringes, and needles may pose an environmental hazard for vaccines using adjuvants or preservatives and for modified live virus vaccines. Instructions for disposal should be included within the vaccine packaging information and based on current environmental regulations in the country of use.

2.3.2. Efficacy requirements

The gaps in knowledge regarding the pathogenesis of VSV infection and the immune mechanism that affords protection against viral infection are limitations for the development and implementation of a validated protocol for an efficacy test. Ideally vaccine efficacy should be estimated in vaccinated animals directly by evaluating their resistance to live virus challenge. Vaccine efficacy should be established for every strain to be authorised for use in the vaccine.

Live reference VSV viruses corresponding to the virus strains circulating in the region are stored at ultralow temperatures. Each challenge virus is prepared as follows. Tongue tissue infected by VSV should be obtained from original field case of VS and received at the Reference Laboratory in glycerol buffer as described in Section B. *Diagnostic Techniques*.

The preparation of cattle challenge virus follows the process described in Chapter 2.1.8 *Foot and mouth disease*, Section B.1.1 *Virus isolation*, with the view of obtaining a sterile 10% suspension in Eagles minimal essential medium with 10% sterile fetal bovine serum.

The preparation of the stock of challenge virus to be aliquoted is prepared starting from lesions collected in two cattle over 6 months of age, previously recognised to be free of VSV antibodies. These animals are tranquillised, for example using xylazine 100 mg/ml (follow instructions for use), then inoculated intradermally (i.d.) in the tongue with the suspension in about 20 sites, 0.1 ml each. The vesiculated tongue tissue is harvested at the peak of the lesions, approximately 2 days later.

A 2% suspension is prepared as above and filtered through a 0.2 µm filter, aliquoted and frozen in the gas phase of liquid nitrogen, and constitutes the stock of challenge virus. The infective titres of this stock are determined both in cell culture ($TCID_{50}$) and in two cattle (BID_{50}). These two cattle that have been tranquilised using xylazine, are injected intradermally in the tongue with tenfold dilutions (1/10 through 1/10,000), using four sites per dilution (Henderson, 1949). The cattle titrations are read 2 days later. Most frequently, titres are above 10^6 $TCID_{50}$ for 0.1 ml and above 10^5 BID_{50} for 0.1 ml calculated using the Spearmann–Kärber method. The dilution for use in cattle challenge test is 10 000 DIB_{50} in a total volume of 4× 0.1 ml by intralingual injection for both the PD_{50} test and the PGP test (House *et al.,* 2003).

i) Vaccination–challenge method

For this experimental method, a group of 12 VSV sero-negative cattle of at least 6 months of age are vaccinated with a bovine dose by the route and in the volume recommended by the manufacturer at day 0 and day 40. These animals and a control group of two non-vaccinated animals are challenged 2 weeks or more after the second vaccination. The challenge strain is a suspension of bovine virus that is fully virulent and appropriate to the virus types in the vaccine under test by inoculating a total of 10,000 BID_{50} intradermally into four sites (0.1 ml per site) on the upper surface of the tongue. Animals are observed at 7–8 days after challenge.

It was proposed that vaccinated animals showing no lesion on the tongue should be considered fully protected. Vaccinated animals showing lesions at one, two, or three inoculation sites should be considered partially protected, and animals showing lesions at four sites are considered not protected (House *et al.,* 2003). Control animals must develop lesions at four sites. Vaccine should fully protect at least nine animals out of 12 vaccinated (75% protection), the remaining animals being partially or not protected. This test gives a certain measure of the protection following the injection of two commercial bovine doses of vaccine in a limited cattle population.

Although the vaccination-challenge method has been described and published (House *et al.*, 2003) data on the validation under field conditions for the efficacy of released vaccine are not available.

ii) Efficacy in other species

Efficacy tests in other target species, such as horses, are not yet described or standardised. In general, a successful test in cattle should be considered to be sufficient evidence of the quality of a VS vaccine to endorse its use in other species.

2.3.3. Duration of immunity

The duration of immunity (D.O.I) of a VS vaccine will depend on the efficacy (formulation and antigen payload). As part of the authorisation/licensing procedure the manufacturer should be required to demonstrate the D.O.I. of a given vaccine by either challenge or the use of a validated alternative test, such as serology at the end of the claimed period of protection.

2.3.4. Stability

The stability of all vaccines including oil emulsion vaccines should be demonstrated as part of the shelf-life determination studies for authorisation/licensing. Vaccines should never be frozen or stored above the target temperature.

i) For animal production

Virus(es) used in vaccine production should be antigenically relevant to virus(es) circulating in the field. A vaccination/challenge study in the species for which the vaccine will be used will indicate the degree of protection afforded by the vaccine. Species used in vaccination/challenge studies should be free of antibodies against vesicular stomatitis. Vaccination/challenge studies should be conducted using virus produced by the intended production method, at the maximum viral passage permitted, and using an experimental animal model. It is necessary to confirm the sensitivity, specificity, reproducibility, statistical significance and confidence level of such experimental model.

Antibody levels after vaccination measured *in vitro* could be used to assess vaccine efficacy provided a statistically significant correlation study has been made. For vaccines containing more than one virus (for example, New Jersey and Indiana-1), the efficacy of the different components of these vaccines must each be established independently and then as a combination in case interference between different viruses exists.

The duration of immunity and recommended frequency of vaccination of a vaccine should be determined before a product is approved. Initially, such information is acquired directly using host animal vaccination/challenge studies. The period of demonstrated protection, as measured by the ability of vaccinates to withstand challenge in a valid test, can be incorporated into claims found on the vaccine label.

If the vaccine is to be used in horses, swine, cattle, or other ruminants destined for market and intended for human consumption, a withdrawal time consistent with the adjuvant used (generally 21 days) should be established by such means as histopathological examination submitted to the appropriate food safety regulatory authorities.

ii) For control

The same principles apply as for animal production usage. In addition, it should be noted that antibody responses in vaccinated animals may not be differentiated from animals exposed to field virus. Therefore, vaccinated animals will need to be clearly identified if serological methods will be used in conjunction with compatible clinical signs to assess field virus exposure.

2.3.5. Stability

Vaccines should be stored at 4–8°C, with minimal exposure to light. The shelf life should be determined by use of the approved potency test (Section C.2.2.4.iii) over the proposed period of viability.

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