

## Section 3.6

## Equidae

## CHAPTER 3.6.9

**EQUINE VIRAL ARTERITIS (INFECTION WITH EQUINE ARTERITIS VIRUS)****SUMMARY**

*Equine viral arteritis (EVA) is a contagious viral disease of equids caused by equine arteritis virus (EAV), an RNA virus classified in the genus, Arterivirus, family Arteriviridae. Equine arteritis virus is found in horse populations in many countries world-wide. Although infrequently reported in the past, confirmed outbreaks of EVA appear to be on the increase.*

**Description of the disease:** *The majority of naturally acquired infections with EAV are subclinical. Where present, clinical signs of EVA can vary in range and severity. The disease is characterised principally by fever, depression, anorexia, dependent oedema, especially of the limbs, scrotum and prepuce in the stallion, conjunctivitis, an urticarial-type skin reaction, abortion and, rarely, a fulminating pneumonia, enteritis or pneumo-enteritis in young foals. Apart from mortality in young foals, the case-fatality rate in outbreaks of EVA is very low. Affected horses almost invariably make complete clinical recoveries. A long-term carrier state can occur in a variable percentage of infected stallions, but not in mares, geldings or sexually immature colts.*

**Identification of the agent:** *EVA cannot be differentiated clinically from a number of other respiratory and systemic equine diseases. Diagnosis of EAV infection is laboratory dependent and based on virus isolation, detection of nucleic acid or viral antigen, or demonstration of a specific antibody response. Detection and identification of EAV nucleic acid in suspect cases of the disease can be attempted using various reverse-transcription polymerase chain reaction (RT-PCR) assays. The identity of isolates of EAV should be confirmed by RT-PCR assay, neutralisation test, or by immunocytochemical methods, namely indirect immunofluorescence or avidin–biotin–peroxidase techniques.*

*Where mortality is associated with a suspected outbreak of EVA, a wide range of tissues should be examined for histological evidence of panvasculitis that is especially pronounced in the small arteries throughout the body. The characteristic vascular lesions present in the mature animal are not a notable feature in EVA-related abortions, diagnosis of which is based on virus isolation, viral nucleic acid detection by RT-PCR or demonstration of EAV antigens by immunohistochemical examination of placental and various fetal tissues.*

**Serological tests:** *A variety of serological tests, including virus neutralisation (VN), complement fixation (CF), indirect fluorescent antibody, agar gel immunodiffusion, the enzyme-linked immunosorbent assay (ELISA), and the fluorescent microsphere immunoassay (MIA) have been used for the detection of antibody to EAV. The tests currently in widest use are the complement-enhanced VN test and the ELISA. The VN test is a very sensitive and highly specific assay of proven value in diagnosing acute infection and in seroprevalence studies. Several ELISAs have been developed. Although none have been as extensively validated as the VN test, some offer comparable specificity and close to equivalent sensitivity. The CF test is less sensitive than either VN test or ELISA, but it can be used for diagnosing recent infection.*

**Requirements for vaccines:** *Two commercial tissue culture derived vaccines are currently available against EVA. One is a modified live virus (MLV) vaccine prepared from virus that has been attenuated for horses by multiple serial transfers in primary equine and rabbit kidney cells and in an equine dermal cell line. It has been confirmed to be safe and protective for stallions and nonpregnant mares. Vaccination of foals less than 6 weeks of age and of pregnant mares in the final 2 months of gestation is not recommended. There is no evidence of back reversion of the vaccine virus to virulence or of recombination with naturally occurring strains of EAV following its use in the field. The second vaccine is an inactivated, adjuvanted product prepared from virus grown in equine cell culture that can be used in nonbreeding and breeding horses. In the absence of appropriate safety data, the vaccine is not currently recommended for use in pregnant mares.*

## A. INTRODUCTION

### 1. Description of disease and aetiology

Equine viral arteritis (EVA) is a contagious viral disease of equids caused by equine arteritis virus (EAV), a positive-sense, single-stranded RNA virus, and the prototype member of the genus *Arterivirus*, family *Arteriviridae*, order *Nidovirales* (Cavanagh, 1997). Only one major serotype of the virus has been identified so far. Epizootic lymphangitis pinkeye, fièvre typhoïde and rotlaufseuche are some of the descriptive terms used in the past to refer to a disease of close clinical resemblance to EVA. The natural host range of EAV would appear to be restricted to equids, although very limited evidence would suggest it may also include new world camelids, *viz.* alpacas and llamas (Weber *et al.*, 2006). The virus does not present a human health hazard (Timoney & McCollum, 1993).

While the majority of cases of acute infection with EAV are subclinical, certain strains of the virus can cause disease of varying severity (Timoney & McCollum, 1993). Typical cases of EVA can present with all or any combination of the following clinical signs: fever, depression, anorexia, leukopenia, dependent oedema, especially of the limbs, scrotum and prepuce of the stallion, conjunctivitis, ocular discharge, supra or periorbital oedema, rhinitis, nasal discharge, a local or generalised urticarial skin reaction, a period of temporary subfertility in acutely affected stallions, abortion, stillbirths and, rarely, a fulminating pneumonia, enteritis or pneumo-enteritis in young foals. Regardless of the severity of clinical signs, affected horses almost invariably make complete recoveries. The case-fatality rate in outbreaks of EVA is very low; mortality is usually only seen in very young foals, particularly those congenitally infected with the virus (Timoney & McCollum, 1993; Vaala *et al.*, 1992), and very rarely in otherwise healthy adult horses.

A variable percentage of acutely infected stallions later become long-term carriers in the reproductive tract and constant semen shedders of the virus (Timoney & McCollum, 1993). The carrier state, which has been shown to be androgen dependant, has been found in the stallion, but not in the mare, gelding or sexually immature colt (Timoney & McCollum, 1993). While temporary down-regulation of circulating testosterone levels using a GnRH antagonist or by immunisation with GnRH would appear to have expedited clearance of the carrier state in some stallions, the efficacy of either treatment strategy has yet to be fully established. Concern has been expressed that such a therapeutic approach could be used to deliberately mask existence of the carrier state.

The gross and microscopic lesions described in fatal cases of EVA reflect the extensive vascular damage caused by the virus (Del Piero, 2000). EAV causes widespread vasculitis, primarily of the smaller arterioles and venules. This gives rise to oedema, congestion and haemorrhages, especially in the subcutis of the limbs and abdomen, and excess peritoneal, pleural and pericardial fluid (Jones *et al.*, 1957). Pulmonary oedema, emphysema and interstitial pneumonia, enteritis and splenic infarcts have been described in fatal cases of EVA in young foals (Del Piero, 2000). Gross lesions are usually absent in cases of abortion and microscopic changes, and if present, are most often seen in the placenta, liver, spleen and lungs of the fetus.

Factors of considered importance in the epidemiology of EVA are phenotypic variation among virus strains, modes of transmission during acute and chronic phases of infection, carrier state in the stallion, nature and duration of acquired immunity and changing trends in the horse industry. EAV is present in the horse population of many countries world-wide (Timoney & McCollum, 1993). There has been an increase in the incidence of EVA in recent years that has been linked to the greater frequency of movement of horses and use of transported semen (Balasuriya *et al.*, 1998). Transmission of EAV can occur by respiratory, venereal and congenital routes. Respiratory spread is most important during the acute phase of the infection. EAV can also be transmitted venereally by the acutely infected stallion or mare and by the carrier stallion.

Current EVA control programmes are aimed at preventing the dissemination of EAV in breeding populations to minimise the risk of abortion outbreaks, deaths in young foals and establishment of the carrier state in colts and stallions (Timoney & McCollum, 1993). Such programmes are based on observance of sound management practices in conjunction with a targeted vaccination program of breeding stallions and sexually immature colts against the disease.

## 2. Differential diagnosis

EVA cannot be differentiated clinically from a number of other respiratory and systemic equine diseases, the most common of which are equine influenza, equine herpesvirus 1 and 4 infections, infection with equine rhinitis A and B viruses, equine adenoviruses and streptococcal infections, with particular reference to purpura haemorrhagica. The disease also has clinical similarities to equine infectious anaemia, equine encephalosis virus infection, African horse sickness fever, cases of Hendra virus infection, Getah virus infection and toxicosis caused by hoary alyssum (*Berteroa incana*) (Timoney & McCollum, 1993).

### B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of equine viral arteritis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection	Efficiency of eradication policies	Confirmation of clinical cases	Prevalence of infection - surveillance	Immune status in individual animals or populations post-vaccination
<b>Identification of the agent<sup>(a)</sup></b>						
Virus isolation	-	+++	-	+++	-	-
PCR	-	+++	-	+++	-	-
<b>Detection of immune response</b>						
AGID	-	-	-	-	-	-
CFT	-	-	-	+++	-	-
ELISA	+	++	+	++	+++	+
VN	+	+++	+	+++	+++	+++

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

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

PCR = polymerase chain reaction; AGID = agar gel immunodiffusion; CFT = complement fixation test;

ELISA = enzyme-linked immunosorbent assay; VN = virus neutralisation.

<sup>(a)</sup>A combination of agent identification methods applied on the same clinical sample is recommended.

Detection and identification of EAV from appropriate clinical and tissue samples can be accomplished by virus isolation in cell culture and by detection of viral nucleic acid using a range of reverse-transcription polymerase chain reaction (RT-PCR) assays. Both diagnostic approaches are appropriate for confirmation of clinical cases of EVA as well as establishing individual animal freedom from EAV infection. In the latter context, virus isolation and RT-PCR assays have been used in surveillance studies and in enabling animal movement to take place. Antigen detection through the use of various immunolabelling techniques also has diagnostic application when examining tissues from suspect cases of EVA abortion, death in young foals or older horses.

Isolation of EAV can be attempted in a limited number of cell lines of which the RK-13 rabbit kidney cell line (ATCC CCL37, or RK13-KY<sup>1</sup>) has proven to be optimal especially when testing stallion semen. Several comprehensive comparison studies have shown virus isolation to be of equivalent sensitivity to RT-PCR for the detection of EAV in clinical and morbid material. Although many isolations of the virus are made in initial passage in cell culture, virus isolation is not a rapid diagnostic test in contrast to certain RT-PCR assays that can be completed in the same day.

A wide variety of RT-PCR assays (single step, nested, real-time) have been developed for EAV detection. Regrettably, very few have been adequately validated and compared with virus isolation for sensitivity and specificity. It is important to emphasise that the choice of reagent kits for both nucleic acid and extraction and amplification in the real-time RT-PCR assay can have a major influence on the overall diagnostic sensitivity and robustness of the assay (Miszczak *et al.*, 2011).

Immunohistochemical testing for EAV antigen in frozen or fixed tissue sections is best accomplished using a monospecific polyclonal serum against the virus or a monoclonal antibody (MAb) directed against the highly conserved nucleocapsid (N) viral protein.

Of the serological tests evaluated for the detection of antibodies to EAV, the complement-enhanced virus neutralisation (VN) test has been proven the most reliable for the diagnosis of acute EAV infection and for serosurveillance studies. Of the numerous enzyme-linked immunosorbent assays (ELISAs) that have been developed, a few offer comparable but not identical sensitivity and specificity to the VN test. A benefit of an EAV ELISA is that it can provide a same-day test result compared with the VN test, which is a 72-hour test. None of the available tests can reliably differentiate antibody titres resulting from natural infection from those due to vaccination.

## 1. Identification of the agent

### 1.1. *In-vitro* culture

In the event of a suspect outbreak of EVA, or when endeavouring to confirm a case of subclinical EAV infection, virus isolation should be attempted preferably from nasopharyngeal or deep nasal swabs, conjunctival swabs, unclotted blood samples, and semen from stallions considered putative carriers of the virus (Timoney & McCollum, 1993). To optimise the chances of virus isolation during an outbreak, relevant specimens should be obtained as soon as possible after the onset of fever in affected horses. In attempting virus isolation from peripheral mononuclear cells (PBMCs), blood should be collected in citrate on ethylenediaminetetraacetic acid (EDTA) anticoagulant. As heparin can inhibit the growth of EAV in rabbit kidney cells (RK-13 cell line), its use as an anticoagulant is contra-indicated as it may interfere with isolation of the virus from whole blood. Where EVA is suspected in cases of mortality in young foals or older animals, virus isolation can be attempted from a variety of tissues, especially the lymphatic glands associated with the alimentary tract and related organs, and also the lungs, liver and spleen (McCollum *et al.*, 1971). In outbreaks of EVA-related abortion and/or cases of stillborn foals, placental and fetal fluids and a wide range of placental, lymphoreticular and other fetal tissues (especially lung) can be productive sources of virus (Timoney & McCollum, 1993).

Swabs for attempted isolation should be immersed in a suitable viral transport medium and these, together with any fluids or tissues collected for virus isolation and/or RT-PCR testing should be shipped either refrigerated or frozen in an insulated container to the laboratory, ideally within 24 hours. If swabs are intended for direct examination by RT-PCR, the swab shaft should not be made of wood, which might contain substances such as preservatives that could interfere with the PCR reaction. Unclotted blood samples must be transported refrigerated but not frozen. Where possible, specimens should be submitted to a laboratory with established competency in testing for this infection.

Nasopharyngeal swabs in transport medium are processed by transferring each into the barrel of a 10 ml syringe, the syringe plunger inserted and whatever fluid can be extracted is collected into a sterile tube. An aliquot of the fluid is passed through a

prefilter and then filtered through a 0.45 µm membrane syringe filter and collected aseptically for subsequent inoculation into cell culture.

Buffy coats can be harvested from unclotted blood by centrifugation at 600 *g* for 15 minutes, and the buffy coat taken off after the plasma has been carefully removed. The buffy coat is then layered onto a PBMC separating solution, Ficoll 1.077, and centrifuged at 400 *g* for 20 minutes. The PBMC interface (without most granulocytes) is washed twice in phosphate buffered saline (300 *g* for 10 minutes) and resuspended in 1 ml of Eagle's minimal essential medium (MEM) containing 2% FCS. A 0.5 ml volume of the rinsed cell suspension is added to monolayers of RK-13 cells in 25 cm<sup>2</sup> flasks or multiwall plates to which maintenance medium is added.

Although reportedly not always successful in natural cases of EAV infection (Timoney & McCollum, 1993), virus isolation should be attempted from clinical specimens or necropsy tissues using rabbit, equine or monkey kidney cell culture (Timoney *et al.*, 2004; Timoney & McCollum, 1993). Selected cell lines, e.g. RK-13 (ATCC CCL-37), LLC-MK<sub>2</sub> (ATCC CCL-7), and primary horse or rabbit kidney cell culture can be used, with RK-13 cells being the cell system of choice (Timoney *et al.*, 2004). Experience over the years has shown that primary isolation of EAV from semen can present more difficulty than from other clinical specimens or from infected tissues unless an appropriate cell culture system is used. Several factors have been shown to influence primary isolation of EAV from semen in RK-13 cells. Higher isolation rates have been obtained using 3- to 5-day-old confluent monolayers, a large inoculum size in relation to the cell surface area in the inoculated flasks or multiwell plates, and most importantly, the incorporation of carboxymethyl cellulose (medium viscosity, 400–800 cps) in the overlay medium. It should be noted that most RK-13 cells, including ATCC CCL-37, are contaminated with bovine viral diarrhoea virus, the presence of which appears to enhance sensitivity of this cell system for the primary isolation of EAV, especially from semen. In the case of specimens of low viral infectivity, isolation rates of EAV may be increased by using RK-13 cells of high passage history<sup>2</sup> (Timoney *et al.*, 2004).

Inoculated cultures are examined daily for the appearance of viral cytopathic effect (CPE), which is usually evident within 2–6 days. In the absence of visible CPE, culture supernatants should be subinoculated on to confluent cell monolayers after 4–7 days. While the vast majority of isolations of EAV are made on the first passage in cell culture, a small minority only become evident on the second or subsequent passages *in vitro* (Timoney & McCollum, 1993). The identity of isolates of EAV can be confirmed by standard or real-time RT-PCR assays (Balasuriya *et al.*, 1998), in a one-way neutralisation test, or by an immunocytochemical method (Little *et al.*, 1995), indirect immunofluorescence (Crawford & Henson, 1973) or avidin-biotin-peroxidase (ABC) technique (Little *et al.*, 1995). A polyclonal rabbit antiserum has been used to identify EAV in infected cell cultures. Mouse monoclonal antibodies (MAbs) to the nucleocapsid protein (N) and major envelope glycoprotein (GP5) of EAV as well as a monospecific rabbit antiserum to the unglycosylated envelope protein (M) (Balasuriya *et al.*, 1998) have also been developed and these can detect various strains of the virus in RK-13 cells as early as 12–24 hours after infection (Balasuriya *et al.*, 1998; Little *et al.*, 1995).

## 1.2. Virus isolation from semen

There is considerable evidence that short- and long-term carrier stallions shed EAV constantly in the semen, but not in respiratory secretions or urine; nor has it been demonstrated in the buffy coat (peripheral blood mononuclear cells) of such animals (Timoney *et al.*, 1987; Timoney & McCollum, 1993). Stallions should first be blood tested using the VN test or an appropriately validated ELISA or other serological test procedure. Virus isolation should be attempted from the semen of stallions serologically positive for antibodies to EAV e.g. VN titre  $\geq 1/4$ , that do not have a certified history of vaccination against EVA, also with confirmation that they were serologically negative (VN titre  $< 1/4$ ) at time of initial vaccination. Virus isolation is also indicated in the case of shipped semen where the serological status and possible vaccination history of the donor stallion is not available. It is recommended that virus isolation from semen be attempted from two samples, which can be collected on the same day, on consecutive days, or after an interval of several days or weeks. There is no evidence that the outcome of attempted virus isolation from particular stallions is influenced by the frequency of sampling, the interval between collections, or time of the year. Isolation of EAV should be carried out preferably on portion of an entire ejaculate collected using an artificial vagina or a condom and a teaser or phantom mare. When it is not possible to obtain semen by this means, a less preferable alternative is to collect a dismount sample at the time of breeding. Care should be taken to ensure that no antiseptics/disinfectants are used in the cleansing of the external genitalia of the stallion prior to collection. Samples should contain the sperm-rich fraction of the ejaculate with which EAV is associated as the virus is not present in the pre-sperm fraction of semen (Timoney *et al.*, 1987; Timoney & McCollum, 1993). Immediately following collection, the semen should be refrigerated on crushed ice or on freezer packs for transport to the laboratory as soon as possible. Where there is likely to be a

delay in submitting a specimen for testing, the semen can be frozen at or below  $-20^{\circ}\text{C}$  for a short period before being dispatched to the laboratory. Freezing a sample does not appear to interfere with isolation of EAV from semen. In situations where it is not feasible to determine the carrier status of a stallion by virus isolation or RT-PCR assay, the stallion can be test bred to two seronegative mares, which are checked for seroconversion to the virus up to 28 days after breeding (Timoney & McCollum, 1993).

It is not possible to reliably determine the carrier status of stallions treated with GnRH antagonist or immunised with GnRH to modify reproductive activity or behaviour, as this may also temporarily interrupt EAV shedding.

### 1.2.1. Test procedure

- i. On receipt in the laboratory, it should be noted whether a semen sample is frozen, chilled or at ambient temperature. Every sample should be checked to ensure that it contains the sperm-rich fraction of the ejaculate. Some stallions can produce large volumes of seminal plasma prior to ejaculating the sperm-rich and gel fractions of semen. Frequently, this pre-sperm fraction contains very few sperm and can be EAV negative even though the stallion is a carrier of EAV (Timoney *et al.*, 1987). To optimise detection of virus, 50  $\mu\text{l}$  of each semen sample should be transferred onto a glass slide, covered with a cover-slip and examined microscopically at a magnification of 100 $\times$  to assess its sperm content. Ejaculates with less than an average of five sperm per ten fields examined should be considered of questionable diagnostic value. It is worth noting however, that the occasional oligospermic stallion can be EAV positive even with a low sperm count. If virus negative on the other hand, the test report on such a stallion should include the qualifier that freedom from EAV cannot be guaranteed based on the low sperm numbers in the sample submitted. Additionally, specimens of ejaculate should be visually inspected and recorded for colour and presence of gross particulate contamination. If a semen specimen is contaminated with blood, which can result from trauma to the external genitalia of the stallion at time of collection, a repeat sample should be requested as testing such a specimen from a serologically positive stallion may compromise the reliability of the virus isolation result due to the EAV antibodies in the serum. Very infrequently, an ejaculate may have a yellowish tinge due to contamination with urine. Such samples may be positive for equine rhinitis A virus.
- ii. Although no longer considered an essential step, pretreatment of semen before inoculation into cell culture by short-term sonication (three 15-second cycles); facilitates effective mixing and dispersion of a sample.
- iii. After removal of culture medium, 3- to 5-day-old confluent monolayer cultures of RK-13 cells, either in 25  $\text{cm}^2$  tissue culture flasks or multiwell plates, are inoculated with serial decimal dilutions (from  $10^{-1}$  to  $10^{-3}$ ) of seminal plasma in tissue culture maintenance medium containing 2% fetal bovine serum and antibiotics. An inoculum of 1 ml per 25  $\text{cm}^2$  flask is used and no fewer than two flasks per dilution of seminal plasma are inoculated. Inoculum size and number of wells inoculated per dilution of a specimen should be pro-rated where multiwell plates are used. Appropriate dilutions of a virus positive control semen sample or virus control of known titre diluted in culture medium should be included in each test.
- iv. The flasks are closed, lids replaced on multiwell plates and inoculated cultures gently rotated to disperse the inoculum over the cell monolayers.
- v. Inoculated cultures are then incubated for 1 hour at  $37^{\circ}\text{C}$  either in an aerobic incubator or an incubator containing a humidified atmosphere of 5%  $\text{CO}_2$  in air, depending on whether flasks or multiwell plates are used.
- vi. Without removing any of the inoculum or washing the cell monolayers, the latter are overlaid with 0.75% carboxymethyl cellulose containing medium with antibiotics.
- vii. The flasks or plates are reincubated at  $37^{\circ}\text{C}$  and checked microscopically for viral CPE, which is usually evident within 2–6 days.
- viii. In the absence of visible CPE, culture supernatants are subinoculated onto 3–5 day-old confluent cell monolayer cultures of RK-13 cells after 5–7 days. After removal of the overlay medium, monolayers are stained with 0.1% formalin-buffered crystal violet solution.

The identity of any virus isolates should be confirmed by standard or real-time RT-PCR (Balasuriya *et al.*, 1998; 2002; Gilbert *et al.*, 1997; Lu *et al.*, 2007; Miszczak *et al.*, 2011; Westcott *et al.*, 2003) by VN, immunofluorescence (Crawford & Henson,

1973) or ABC technique, using a monospecific antiserum to EAV or MAbs to the structural proteins, N or GP5 of the virus (Balasuriya *et al.*, 1998; Del Piero, 2000; Little *et al.*, 1995).

In the one-way neutralisation test, serial decimal dilutions of the virus isolate are tested against a neutralising MAb or monospecific antiserum prepared against the prototype Bucyrus strain of EAV (ATCC VR 796) and also a serum negative for neutralising antibodies to the virus. Corresponding titrations of the prototype Bucyrus virus with the same reference antibody reagents are included as test controls. The test is performed in either 25 cm<sup>2</sup> tissue culture flasks or multiwell plates. Appropriate quantities of the known EAV positive and negative antibody reagents are inactivated for 30 minutes in a water bath at 56°C and diluted 1/4 in phosphate buffered saline, pH 7.2; then 0.3 ml of diluted antibody reagent is dispensed into five tubes for each isolate to be tested. Serial decimal dilutions (from 10<sup>-1</sup> to 10<sup>-5</sup>) of each virus isolate are made in Eagle's MEM containing 10% fetal bovine serum, antibiotics and 10% freshly diluted guinea-pig complement. Then, 0.3 ml of each virus dilution is added to the tubes containing positive and negative antibody reagents. The tubes are shaken and the virus/antibody mixtures are incubated for 1 hour at 37°C. The mixtures are then inoculated onto 3- to 5-day-old confluent monolayer cultures of RK-13 cells, either in 25 cm<sup>2</sup> flasks or multiwell plates, using two flasks or wells per virus dilution. Each flask is inoculated with 0.25 ml of virus/antibody mixture; the inoculum size is pro-rated where multiwell plates are used. Inoculated flasks or plates are incubated for 2 hours at 37°C, gently rocking after 1 hour to disperse the inoculum over the cell monolayers. Without removing any of the inoculum or washing the cell monolayers, the latter are overlaid with 0.75% carboxymethyl cellulose containing medium and incubated for 4–5 days at 37°C, either in an aerobic incubator or an incubator containing a humidified atmosphere of 5% CO<sub>2</sub> in air. After removal of the medium, monolayers are stained with 0.1% formalin-buffered crystal violet solution. Plaques are counted and the virus infectivity titre is determined both in the presence and absence of EAV antibodies using the Spearman–Kärber method. Confirmation of the identity of an isolate is based on a reduction in plaque count of at least 10<sup>2</sup> logs of virus in the presence of antibody positive serum against the Bucyrus strain of EAV.

The vast majority of EAV isolates from carrier stallions are made in the first passage in cell culture using the described test procedure (Timoney & McCollum, 1993). The occurrence of nonviral cytotoxicity or bacterial contamination of specimens is not a significant problem when attempting isolation of this virus from stallion semen. Nonviral cytotoxicity, if observed, usually affects monolayers inoculated with the 10<sup>-1</sup> and, much less frequently, the 10<sup>-2</sup> dilution of seminal plasma. Treatment of seminal plasma with polyethylene glycol (Mol. wt 6000) prior to inoculation has been used with some success in overcoming this problem (Fukunaga *et al.*, 2000). The method described involves the addition of polyethylene glycol to the 10<sup>-1</sup> to 10<sup>-3</sup> dilutions of seminal plasma to give a final concentration of 10% in each dilution. The mixtures are held overnight at 4°C with gentle stirring, after which they are centrifuged at 2000 *g* for 30 minutes and the supernatants are discarded. The precipitates are suspended in cell culture maintenance medium to one-tenth the volume of the original dilutions and the mixtures are homogenised. They are then centrifuged at 2000 *g* for 30 minutes and the supernatants are taken off and used for inoculation. There is no evidence to indicate that pretreatment of seminal plasma in this manner reduces sensitivity of the virus isolation procedure (Fukunaga *et al.*, 2000). Where bacterial contamination of a sample is a problem, it is preferable to request a repeat semen collection from the individual stallion. If this is not possible, an attempt can be made to control the contamination by pre-treatment of the sample with antibiotic containing viral transport medium, holding overnight at 4°C followed by ultracentrifugation and resuspension of the pellet before diluting and inoculating the specimen into cell culture.

There have been two reports of failure to isolate EAV from individual stallions whose semen was positive for viral nucleic acid on RT-PCR assay. In one case at least, failure to detect infectious virus may well have been the result of a very high level of neutralising activity in the seminal plasma of the stallion, emphasising the value of RT-PCR as an adjunct to virus isolation for detection of EAV.

### 1.3. Antigen detection

Where mortality is associated with a suspected outbreak of EVA, a wide range of tissues should be examined for histological evidence of panvasculitis that is especially pronounced in the small arteries and venules throughout the body, particularly in the caecum, colon, spleen, associated lymphatic glands and adrenal cortex (Crawford & Henson, 1973; Del Piero, 2000; Jones *et al.*, 1957). The presence of a disseminated necrotising arteritis involving endothelial and medial cells of affected vessels is considered a pathognomonic feature of EVA. The characteristic vascular lesions present in the mature animal are not, however, a prominent feature in many cases of EAV-related abortion.

EAV antigen can be identified in various tissues of EVA-affected animals either in the presence or absence of lesions (Del Piero, 2000). Antigen has been demonstrated in lung, heart, liver and spleen and the placenta of aborted fetuses (Del Piero, 2000). Immunohistochemical examination of biopsied skin specimens has also been investigated as a means of confirming acute EAV infection. Though of some value, it is not entirely reliable for the diagnosis of the disease. Viral antigen can be detected within the cytoplasm of infected cells by immunofluorescence using conjugated equine polyclonal anti-EAV serum (Crawford & Henson, 1973), or by the ABC technique using mouse MAbs to the GP5 or N proteins of the virus (Del Piero, 2000).

#### 1.4. Molecular methods

The standard two-step RT-PCR, single-step RT-PCR, RT-nested PCR, and real-time RT-PCR (rRT-PCR) assays have become widely accepted as an alternative or adjunct to virus isolation in cell culture for the detection of EAV in diagnostic materials. The RT-PCR-based assays provide a means of identifying virus-specific RNA in clinical specimens, namely nasopharyngeal or nasal swab filtrates, buffy coats, raw and extended semen and urine, and in post-mortem tissue samples (Balasuriya *et al.*, 2002; Gilbert *et al.*, 1997; Lu *et al.*, 2007; Miszczak *et al.*, 2011; Westcott *et al.*, 2003). Standard, single-step RT-PCR, RT-nested PCR (RT-nPCR), and one tube TaqMan® rRT-PCR assays have been developed and evaluated for the detection of various strains of the virus in tissue culture fluid, semen and nasal secretions (Balasuriya *et al.*, 2002; Gilbert *et al.*, 1997; Lu *et al.*, 2007; Miszczak *et al.*, 2011; Westcott *et al.*, 2003). These assays targeted six different open reading frames (ORFs) in the EAV genome (ORFs 1b, 3–7). However, there is considerable variation in the sensitivity and specificity among RT-PCR assays incorporating different primer pairs targeting various ORFs. Results comparable to virus isolation have been obtained with some but not all standard single-step RT-PCR, two-step RT-PCR, RT-nPCR or one tube TaqMan® rRT-PCR assays (Balasuriya *et al.*, 2002; Gilbert *et al.*, 1997; Lu *et al.*, 2007; Miszczak *et al.*, 2011). Compared with traditional virus isolation, these RT-PCR-based assays are frequently more sensitive and considerably more rapid to perform, the majority taking less than 24 hours to complete. In addition, RT-PCR assays have the advantage of not requiring viable virus for performance of the test. The one-tube rRT-PCR assay for EAV provides a simple, rapid and reliable method for the detection and identification of viral nucleic acid in equine semen and tissue culture fluid (Balasuriya *et al.*, 2002; Lu *et al.*, 2007; Miszczak *et al.*, 2011). However, there is evidence to indicate that the choice of commercial kit used for nucleic acid extraction and also for amplification can have a major influence on the overall diagnostic sensitivity and robustness of the assay (Miszczak *et al.*, 2011). This was demonstrated using a magnetic-bead-based nucleic acid extraction method in combination with specific commercial RT-PCR kits. The one tube rRT-PCR has the following important advantages over the standard two-step RT-PCR: 1) eliminating the possibility of cross contamination between samples with previously amplified products as the sample tube is never opened; and 2) reducing the chance of false-positive reactions where the rRT-PCR product is detected with a sequence-specific probe. Because of the high sensitivity of the RT-PCR assay, however, and in the absence of appropriate safeguards in the laboratory, there is the potential for cross-contamination between samples, giving rise to false-positive results. For example, the RT-nPCR assay, while it provides enhanced sensitivity for the detection of EAV, it also increases the likelihood of false-positive results. The risk of cross-contamination is greater using the RT-nPCR assay because of the second PCR amplification step involving the product from the first RT-PCR reaction. To minimise the risk of cross-contamination, considerable care needs to be taken, especially during the steps of RNA extraction and reaction setup. Relevant EAV positive and negative template controls and, where appropriate, nucleic acid extracted from the tissue culture fluid of uninfected cells, need to be included in each RT-PCR assay. Thus, in most circumstances, use of the single-step RT-PCR or one tube rRT-PCR assay will largely circumvent the problems associated with cross contamination.

Primer selection is critical to the sensitivity of the RT-PCR assay with primers (and probe in the case of the rRT-PCR assay) preferably designed from the most conserved region(s) of the EAV genome. Comparative nucleotide sequence analysis has shown that ORF 1b (encodes the viral polymerase), ORF 6 (M protein) and 7 (N protein) are more conserved than the other ORFs among EAV strains so far analysed from North America and Europe (Balasuriya *et al.*, 2002; Lu *et al.*, 2007; Miszczak *et al.*, 2011; Westcott *et al.*, 2003). The most conserved gene among different strains of EAV is ORF7 and primers specific for ORF7 (and probe for rRT-PCR) have detected a diversity of strains of the virus of European and North American origin (Balasuriya *et al.*, 2002; Lu *et al.*, 2007). Furthermore, the use of multiple primer pairs specific for different ORFs 1b ([forward: 5'-GAT-GTC-TAT-GCT-CCA-TCA-TT-3' and reverse: 5'-GGC-GTA-GGC-TCC-AAT-TGA-A-3']) and/or [forward: 5'-CCT-GAG-ACA-CTG-AGT-CGC-GT-3' and reverse: 5'-CCT-GAT-GCC-ACA-TGG-AAT-GA-3']) (Gilbert *et al.*, 1997), ORF 6 ([forward: 5'-CTG-AGG-TAT-GGG-AGC-CAT-AG-3' and reverse: 5'-GCA-GCC-AAA-AGC-ACA-AAA-GC-3']) and ORF 7 ([forward: 5'-ATG-GCG-TCA-AGA-CGA-TCA-CG-3' and reverse: 5'-AGA-ATA-TCC-ACG-TCT-TAC-GGC-3']) markedly increases the likelihood of detecting North American and European strains of EAV in the RT-PCR assay. The two primer pairs specific for ORF 1b are suitable for use in a rRT-PCR assay (Gilbert *et al.*, 1997). While the RT-PCR has been found to be

highly sensitive for viral nucleic acid detection in raw semen, there is evidence that it is not of equivalent reliability when testing cryopreserved semen of very low virus infectivity (Zhang *et al.*, 2004).

In addition to the foregoing RT-PCR assays, 2 TaqMan® fluorogenic probe-based one-tube rRT-PCR assays have been described for the detection of EAV nucleic acid (Balasuriya *et al.*, 2002); primers ([forward: 5'-GGC-GAC-AGC-CTA-CAA-GCT-ACA-3', reverse: 5'-CGG-CAT-CTG-CAG-TGA-GTG-A-3'] and probe [5'-FAM-TTG-CGG-ACC-CGC-ATC-TGA-CCA-A-TAMRA-3'] and (Westcott *et al.*, 2003); primers [forward: 5'-GTA-CAC-CGC-AGT-TGG-TAA-CA-3', reverse: 5'-ACT-TCA-ACA-TGA-CGC-CAC-AC-3'] and probe [5'-FAM-TGG-TTC-ACT-CAC-TGC-AGA-TGC-CGG-TAMRA-3']. It should be noted, however, that genomic variation among field isolates of EAV could reduce the sensitivity of both RT-PCR and rRT-PCR assays, even when the primers and probe are based on the most conserved region of the EAV genome (ORF 7 [Lu *et al.*, 2007]). Phylogenetic studies of strains of EAV from certain regions/countries have confirmed the existence of clusters of isolates more closely related to one another than to virus strains of disparate geographic backgrounds (Mankoc *et al.*, 2007). Under such circumstances, validated primers besides those already recommended may be more suitable for detection of these genomically distinct strains of EAV.

In the absence of widespread agreement on a universal primer set for EAV, and as no RT-PCR assay can determine the actual infectivity of a sample, there is a value to performing virus isolation in conjunction with RT-PCR or rRT-PCR for the identification of virus in clinical or post-mortem specimens and where indicated, genomic and phenotypic analysis of viral isolates.

Strains of EAV isolated from different regions of the world have been classified into different phylogenetic groups by sequence analysis of the GP3, GP5 and M envelope protein genes (ORFs 3, 5 and 6 respectively) and the nucleocapsid (N) protein gene (ORF 7 [Balasuriya *et al.*, 1998; Zhang *et al.*, 2010]). The GP5 gene has been found to be most useful and reliable for this purpose. The relationships between strains demonstrated by nucleotide sequencing are a useful molecular epidemiological tool for tracing the origin of outbreaks of EVA (Balasuriya *et al.*, 1998; Zhang *et al.*, 2010).

## 2. Serological tests

A variety of serological tests have been investigated for their ability to detect antibodies to EAV. These include the neutralisation (microneutralisation [Senne *et al.*, 1985] and plaque reduction [McCollum, 1970]), the complement fixation (CF) test (Fukunaga & McCollum, 1977), the indirect fluorescent antibody test (Crawford & Henson, 1973), the agar gel immunodiffusion (Crawford & Henson, 1973), the ELISA (Cho *et al.*, 2000; Hedges *et al.*, 1998; Kondo *et al.*, 1998; Nugent *et al.*, 2000) and the fluorescent microsphere immunoassay (MIA) (Go *et al.*, 2008).

Interestingly, only one major serotype of EAV represented by the prototype Bucyrus strain (ATCC VR 796) has been recognised so far (McCollum, 1970; Timoney & McCollum, 1993). Antiserum to unpurified EAV has been prepared in horses and in rabbits using conventional immunisation protocols. Mouse MAbs and monospecific rabbit antibodies have also been developed to the nucleocapsid protein (N) major envelope glycoprotein (GP5), and unglycosylated envelope protein (M) of EAV (Balasuriya *et al.*, 1997).

### 2.1. Complement-enhanced microneutralisation test

The complement-enhanced microneutralisation is currently the test in widest international use to diagnose EAV infection, carry out seroprevalence studies, and test horses for movement. It has also been used to screen fetal heart blood as a means of retrospectively diagnosing cases of EVA-related abortion. Neutralising antibodies to EAV persist for several years after natural infection or vaccination with the modified live vaccine against EVA (Timoney & McCollum, 1993).

### 2.2. Virus neutralisation test

The VN test is used for diagnostic purposes to confirm infection in suspect cases/outbreaks of EVA and to screen horses e.g. stallions, for evidence of EAV infection. The test procedure currently in widest use is that developed by the National Veterinary Service Laboratories of the United States Department of Agriculture (Senne *et al.*, 1985). It is important to obtain a sterile blood sample as bacterial contamination of serum can interfere with the test result. It is recommended that the test be carried out in RK-13 cells using the approved CVL-Bucyrus (Weybridge) strain of EAV as reference virus<sup>[3]</sup> (Edwards *et al.*, 1999). Although originally derived from the prototype Bucyrus virus, the passage history of the CVL (Weybridge) strain is not fully documented. Stocks of the reference virus are grown in the RK-13 cell line, clarified of cellular debris by low-speed centrifugation and stored in aliquots at -70°C. Several frozen aliquots are thawed and the infectivity of the stock virus is

determined by titration in RK-13 cells. The sensitivity of the VN test for detection of antibodies to EAV can be significantly influenced by several factors, especially the source and passage history of the strain of virus used (Edwards *et al.*, 1999). The CVL-Bucyrus (Weybridge) strain and the highly attenuated MLV vaccine strain of EAV are of comparable sensitivity for detecting low-titred positive sera, especially from EVA-vaccinated horses. Efforts are continuing to bring about greater uniformity in the testing protocol and serological results among laboratories providing the VN or other comparable serological assays for this infection. WOAHA Approved Standard Sera for EAV are available<sup>[4]</sup> and these can facilitate international standardisation of the microneutralisation test and ELISA.

### 2.2.1. Test procedure

- i. Sera are inactivated for 30 minutes in a water bath at 56 °C (control sera, only once).
- ii. Serial twofold dilutions of the inactivated test sera in serum-free cell culture medium (25 µl volumes) are made in a 96-well, flat-bottomed, cell-culture grade microtitre plate starting at a 1/2 serum dilution and using duplicate rows of wells for each serum to be tested. Most sera are screened initially at a 1/4 and 1/8 serum dilution (i.e. final serum dilution after addition of an equal volume of the appropriate dilution of stock virus to each well). Positive samples at the 1/8 dilution can, if desired, be retested and titrated out for end-point determination. Individual serum controls, together with negative and known low- and high-titred positive control sera must also be included in each test.
- iii. A dilution of stock virus made up to contain from 100 to 300 TCID<sub>50</sub> (50% tissue culture infective dose) per 25 µl is prepared using as diluent, serum-free cell culture medium containing antibiotics and fresh guinea-pig or rabbit complement at a final concentration of 10%.
- iv. 25 µl of the appropriate dilution of stock virus is added to every well containing 25 µl of each serum dilution, except the test serum toxicity control wells and cell control wells on each plate.
- v. A virus back titration of the working dilution of stock virus is included, using four wells per tenfold dilution, to confirm the validity of the test results.
- vi. The plates are covered and shaken gently to facilitate mixing of the serum/virus mixtures.
- vii. The plates are incubated for 1 hour at 37 °C in a humid atmosphere of 5% CO<sub>2</sub> in air.
- viii. A suspension of cells from 3- to 5-day-old cultures of RK-13 cells are prepared using a concentration that will ensure confluent monolayers in the microtitre plate wells within 18–24 hours after seeding.
- ix. 100 µl of cell suspension is added to every well, the plates covered with plate lids or sealed with tape and shaken gently.
- x. The plates are incubated at 37 °C in a humid atmosphere of 5% CO<sub>2</sub> in air.
- xi. The plates are read microscopically for nonviral CPE after 12–18 hours and again for viral CPE after 48–72 hours' incubation. The validity of the test is confirmed by establishing that the working dilution of stock virus contained 30–300 TCID<sub>50</sub> virus and that the positive serum controls are within 0.3 log<sub>10</sub> units of their predetermined titres.

A serum dilution is considered to be positive if there is an estimated 75% or greater reduction in the amount of viral CPE in the serum test wells compared with that present in the wells of the lowest virus control dilution. End-points are then calculated using the Spearman–Kärber method. A titre of 1/4 or greater is considered to be positive. A negative serum should only have a trace (less than 25%) or no virus neutralisation at the lowest dilution tested. Antibody titres may, on occasion, be difficult to define as partial neutralisation may be observed over a range of several serum dilutions. Not infrequently, sera will be encountered that give rise to toxic changes in the lower dilutions tested. In such cases it may not be possible to establish whether the sample is negative or a low-titred positive. The problem may be overcome by retesting the toxic sample using microtitre plates with confluent monolayers of RK-13 cells that had been seeded the previous day. Also, the toxicity in serum samples can be reduced or eliminated if the sample is adsorbed with a packed suspension of RK-13 cells prior to testing or by substituting rabbit in place of guinea-pig complement in the virus diluent. It would appear that there is more than one type of cytotoxicity in sera. Vaccination status for equine herpesviruses should be considered when evaluating sera causing non-viral cytotoxicity. One of the equine herpesvirus vaccines currently available in Europe has been shown to stimulate antibodies to rabbit kidney cells used in the vaccine production. These, in turn, can give rise to cytotoxicity, usually in the 1/4 and/or 1/8 serum but sometimes at higher dilutions, and cause difficulties in interpretation of the test results (Newton *et al.*, 2004).

### 2.3. Enzyme-linked immunosorbent assay

A number of direct or indirect ELISAs have been developed for the detection of antibodies to EAV (Cho *et al.*, 2000; Hedges *et al.*, 1998; Kondo *et al.*, 1998; Nugent *et al.*, 2000). These have been based on the use of purified virus or recombinant-derived viral antigens. The usefulness of earlier assays was compromised by the frequency of false-positive reactions. The latter were associated with the presence of antibodies to various tissue culture antigens in the sera of horses that had been vaccinated with tissue-culture-derived antigens. Identification of the importance of the viral GP5 protein in stimulation of the humoral antibody response to EAV led to the development of several ELISAs that employ a portion of, or the entire recombinant protein produced in a bacterial or baculovirus expression system (Cho *et al.*, 2000; Hedges *et al.*, 1998). Most recently, an ovalbumin-conjugated synthetic peptide representing amino acids 81–106 of the GP5 protein has been used (Nugent *et al.*, 2000). Some of these assays appear to offer nearly comparable sensitivity and specificity to the VN test and may detect EAV-specific antibodies prior to a positive reaction being obtainable in the VN test. False-negative reactions can occur, however, with some of these assays. Screening a random peptide-phage library with polyclonal sera from EAV-infected horses led to the identification of ligands, which were purified and used as antigen in an ELISA for EAV. No correlation was found, however, between absorbency values obtained with this assay and neutralising antibody titres, indicating that the antibodies being detected were largely against nonsurface epitopes of the virus. An ELISA based on the use of a combination of the GP5, M or N structural proteins of EAV expressed from recombinant baculoviruses successfully detected viral antibody in naturally or experimentally infected horses but not in EVA-vaccinated animals (Hedges *et al.*, 1998). Of major importance with respect to any GP5 protein-based ELISA for EAV is the fact that test sensitivity will vary depending on the ectodomain sequence(s) of this viral protein used in the assay. Considerable amino acid sequence variation within this domain has been found between isolates of EAV. To maximise sensitivity of a GP5-based ELISA, it may be necessary to include multiple ectodomain sequences representative of known phenotypically different isolates of EAV rather than depend on a single ectodomain sequence. Two more recently described ELISAs appear to offer most promise as reliable serodiagnostic tests for EAV infection (Cho *et al.*, 2000; Nugent *et al.*, 2000). A blocking ELISA involving MAbs produced against the GP5 protein was reported to have a sensitivity of 99.4% and a specificity of 97.7% compared with the VN test (Cho *et al.*, 2000). Another assay, a GP5 ovalbumin-conjugated synthetic peptide ELISA was shown to have a sensitivity and specificity of 96.75% and 95.6%, respectively, using a panel of 400 VN positive sera and 400 VN negative samples (Nugent *et al.*, 2000). Of the number of ELISAs that have been developed (Cho *et al.*, 2000; Hedges *et al.*, 1998; Kondo *et al.*, 1998; Nugent *et al.*, 2000), few, if any, have been as extensively validated as the VN test, though some would appear to offer nearly comparable sensitivity and specificity (Cho *et al.*, 2000; Hedges *et al.*, 1998; Nugent *et al.*, 2000). It should be noted that unlike the VN test, a positive reaction in the ELISA is not necessarily reflective of the protective immune status of an individual horse to EAV as both non-neutralising and neutralising antibodies are involved.

#### 2.4. Complement fixation test

The CF test has been used in the past for diagnosing recent infection with EAV based on the fact that complement-fixing antibodies are relatively short-lived in duration (Fukunaga & McCollum, 1977). The test has been very largely superseded by the VN test and different ELISAs for carrying out serosurveillance studies and testing horses for movement.

#### 2.5. Fluorescent-microsphere immunoassay

A fluorescent-MIA has been developed to detect equine antibodies to the major structural proteins of EAV (Go *et al.*, 2008). It was based on cloning and expressing full-length individual major proteins, (GP5, M, N), as well as partial sequences of each structural protein and including these in separate assays. The different immunassays were analysed with a Luminex instrument. A partial GP5 protein based assay provided the best results, with sensitivity and specificity values of 92.6% and 93.9% respectively, compared with the VN test.

### C. REQUIREMENTS FOR VACCINES

#### 1. Background

A number of experimental and commercial vaccines have been developed against EVA. Currently, there are two commercially available vaccines, both tissue-culture derived. The first is a modified live virus (MLV) vaccine and the second an inactivated adjuvanted vaccine. The MLV vaccine is commercially available in the USA and Canada. It has also been used under ministerial control in Argentina and in New Zealand. The inactivated vaccine is licensed for commercial use in certain European countries, including Denmark, France, Germany, Hungary, Ireland, Sweden and the United Kingdom. Indications for use of these vaccines are to prevent outbreaks of EVA,

including abortion in pregnant mares and establishment of the carrier state in the stallion. Since the carrier stallion is considered the principal reservoir of EAV, reduction in the carrier population would over time result in greater control over EVA and ultimately could contribute to eradication of the disease in certain countries. The MLV vaccine is prepared from virus that has been attenuated for horses by multiple serial transfers in primary equine and rabbit cells and in an equine dermal cell line (Doll *et al.*, 1968; McCollum, 1970). This vaccine is licensed for use in stallions, nonpregnant mares and in nonbreeding horses. Whereas nonbreeding horses can be vaccinated at any time, stallions and mares should be vaccinated not less than 3 weeks prior to breeding. The vaccine is not recommended for use in pregnant mares, especially in the last 2 months of gestation, nor in foals under 6 weeks of age unless in the face of significant risk of exposure to natural infection.

The second commercially available vaccine against EVA is an inactivated product prepared from virus grown in equine cell culture, which is filtered, chemically inactivated and then combined with a metabolisable adjuvant. This vaccine is licensed for use in nonbreeding and breeding horses. In the absence of appropriate safety data, the vaccine is currently not recommended for use in pregnant mares.

An additional inactivated vaccine against EVA has been developed in Japan and is kept in storage for distribution should an outbreak of EVA occur in that country. It is an aqueous formalin-inactivated vaccine that has been shown to be safe and effective for use in nonbreeding and breeding horses. For optimal immunisation with this vaccine, horses require a primary course of two injections given at an interval of 4 weeks, with a booster dose administered every 6–12 months. As the vaccine is not commercially available, no details can be provided on its production.

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

### 2.1. Characteristics of the seed

Both MLV and inactivated commercial vaccines are derived from the prototype Bucyrus strain of EAV (ATCC VR 796), an experimentally derived variant of a foetal lung isolate recovered during an extensive outbreak of respiratory disease and abortion near Bucyrus, Ohio, USA, in 1953 (Doll *et al.*, 1957). Available evidence points to the existence of only one major serotype of the virus, and strain variation is not considered to be of significance in relation to vaccine efficacy (McCollum, 1970; Timoney & McCollum, 1993).

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

In the case of the MLV vaccine, the prototype virus (ATCC VR 796) was attenuated by serial passage in primary cultures of horse kidney (HK-131), rabbit kidney (RK-111), and a diploid equine dermal cell line, ATCC CCL57 (ECID-24) (Doll *et al.*, 1968; McCollum, 1970). The indications from use of this vaccine are that the virus is safe and immunogenic between its 80th and 111th passage in primary rabbit kidney cells (Doll *et al.*, 1968; McCollum, 1970).

The inactivated adjuvanted vaccine is prepared from the unattenuated prototype Bucyrus strain of EAV (ATCC VR 796) that has been plaque purified and in its fourth serial passage in the diploid equine dermal cell line (ECID-4). After growth in cell culture, the virus is then purified by filtration before being chemically inactivated and adjuvanted.

The virus for both MLV and inactivated vaccines should be grown in a stable cell culture system, such as equine dermal cells, using an appropriate medium supplemented with sterile bovine serum or bovine serum albumin as replacement for bovine serum in the growth medium. Cell monolayers should be washed prior to virus inoculation to remove traces of bovine serum. Extensive virus growth as evidenced by the appearance of cytopathic changes in 80–100% of the cells should be obtained within 2–3 days. Lots of master seed virus for each vaccine are maintained in liquid nitrogen or its equivalent.

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

Tests for sterility, purity and freedom of vaccines from contamination with extraneous agents can be found in Chapter [1.1.9 Tests for sterility and freedom from contamination of biological materials intended for veterinary use](#).

#### 2.1.3. Validation as a vaccine strain

In the case of both MLV and inactivated vaccines, the respective virus strains should be grown in an appropriate cell culture system that has been officially approved for vaccine production and confirmed to be free from extraneous bacteria, fungi, mycoplasmas and viruses (Moore, 1986). The identity of the vaccine virus in the master seed should be confirmed by neutralisation with homologous anti-EAV serum. Incomplete neutralisation of EAV by homologous horse or rabbit antisera has been scientifically documented (Moore, 1986; Senne *et al.*, 1985) and is a problem when screening master seed virus for extraneous viruses and when attempting to confirm the identity of the vaccine virus. The problem has been circumvented by reducing the infectivity titre of the master seed virus below that required for seed virus production before conducting a neutralisation test on the diluted virus. Virus/serum mixtures are tested for residual live virus by serial passage in cell culture. No evidence of cytopathic viruses, haemadsorbing viruses, or noncytopathic strains of bovine virus diarrhoea virus should be found, based on attempted virus isolation in cell culture. If cells of equine origin are used, they should be confirmed to be free from equine infectious anaemia virus. Conventional technologies such as PCR and antigen-capture ELISAs are now more commonly used than virus isolation in screening for adventitious agents.

## 2.2. Method of manufacture

### 2.2.1. Procedure

Both the MLV and inactivated vaccines are produced by cultivation of the respective seed viruses in an equine dermal cell system. Cell monolayers should be washed prior to inoculation with seed virus to remove traces of bovine serum in the growth medium. Inoculated cultures should be maintained on an appropriate maintenance medium. Harvesting of infected cultures should take place when almost the entire cell sheet shows the characteristic CPE. Supernatant fluid and cells are harvested and clarified of cellular debris and unwanted material by filtration. In the case of the inactivated vaccine, the purified virus is then chemically inactivated and adjuvanted with a metabolisable adjuvant. The preservatives added to the MLV and inactivated vaccines are neomycin, polymyxin B and amphotericin B.

### 2.2.2. Requirements for ingredients

Refer to Chapter 1.1.8 *Principles of veterinary vaccine production*, the focus of which is on products of biological origin of negligible risk.

### 2.2.3. In-process controls

The MLV and inactivated vaccines should be produced in a stable cell line that has been tested for identity and confirmed to be free from contamination by bacteria, fungi, mycoplasmas or other adventitious agents. In addition to the preproduction testing of the master seed virus for each vaccine and the cell line for adventitious contaminants, the cell cultures infected with the respective vaccine viruses should be examined macroscopically for evidence of microbial growth or other extraneous contamination during the incubation period. If growth in a culture vessel cannot be reliably determined by visual examination, subculture, microscopic examination, or both should be carried out.

### 2.2.4. Final product batch tests

- i. Sterility  
Tests for sterility and freedom from contamination of biological materials intended for veterinary use can be found in chapter 1.1.9. In the case of both MLV and inactivated vaccines, each production lot of vaccine should be checked for extraneous bacterial, fungal and mycoplasmal contaminants.
- ii. Safety  
The vaccine should be safety tested by the intramuscular inoculation of at least two horses seronegative for neutralising antibodies to EAV with one vaccine dose of lyophilised virus each (Moore, 1986). None of the inoculated horses should develop any clinical signs of disease other than mild pyrexia during the ensuing 2-week observation period. Transient local reactions may be observed in less than 10% of horses inoculated with either vaccine. In addition, nasopharyngeal swabs should be collected daily from each horse for attempted virus isolation; white blood cell counts and body temperatures should also be determined on a daily basis. No significant febrile or haematological changes should supervene following vaccination (Timoney & McCollum, 1993). Limited shedding of vaccine virus by the respiratory route and

in semen may be demonstrated in the occasional horse within the first 7 days after vaccination. There is no evidence of persistence of the vaccine virus in the reproductive tract of vaccinated stallions (Timoney & McCollum, 1993).

To ensure complete inactivation of the vaccine virus, each serial lot of the inactivated vaccine should be checked for viable virus by three serial passages in equine dermal cells and by direct fluorescent antibody staining with specific EAV conjugate before being combined with adjuvant. This should be followed by a safety test in guinea-pigs and mice.

iii. Batch potency

Potency of the vaccine in the final containers is determined by plaque infectivity assay in monolayer cultures of equine dermal cells and by a vaccination challenge test in horses (Moore, 1986). The vaccine must be tested in triplicate in cell culture, the mean infectivity titre calculated and the dose rate determined on the basis that each dose of vaccine shall contain not less than  $3 \times 10^4$  plaque-forming units of attenuated EAV. The *in-vivo* potency of the MLV and inactivated vaccines is evaluated in a single vaccination challenge test using 17–20 vaccinated and 5–7 control horses or in two tests each comprising ten vaccinates and five controls. The viral antigen concentration in the inactivated vaccine is over one-thousand times the concentration of viral antigen present in the MLV vaccine.

### 2.3. Requirements for authorisation/registration/licensing

#### 2.3.1. Manufacturing process

For vaccine registration, all relevant details concerning manufacture of the vaccine and quality control section should be submitted to the authorities. This information shall be provided from three consecutive batches with a volume of not less than 1/3 of the typical industrial batch volume.

#### 2.3.2. Safety requirements

The manufacturer of the MLV recommends a single dose of vaccine administered intramuscularly for primary vaccination followed by annual revaccination. The recommended vaccination regimen for the inactivated vaccine, which should also be administered by the intramuscular route, is a primary course of two vaccinations 3–6 weeks apart, followed by revaccination every 6 months.

i. Target and non-target animal safety

The MLV vaccine is considered safe for stallions and nonpregnant mares. There is no evidence to indicate that the vaccine virus can establish the carrier state in the vaccinated stallion. The MLV vaccine is not recommended for use in pregnant mares or in foals less than 6 weeks of age. Although contra-indicated by the manufacturer, this vaccine has been used in pregnant mares in the first two trimesters without any adverse sequelae. There is the risk of abortion in mares vaccinated within the last two months of gestation. The inactivated vaccine is safe for use in non-breeding and breeding animals. In the absence of appropriate safety data, the vaccine is not currently recommended for use in pregnant mares.

ii. Reversion-to-virulence for attenuated live vaccines and environmental considerations

Both experimental and extensive field studies conducted since the MLV vaccine was first released commercially in 1985, have failed to provide any evidence of back reversion to virulence or of recombination with naturally occurring strains of EAV (Timoney & McCollum, 1993).

iii. Precautions

The manufacturer of both the MLV and inactivated vaccines provides adequate information in the respective vaccine inserts as to the recommended usage of each vaccine, including certain contra-indications in the case of the MLV vaccine. Neither vaccine is harmful to vaccinators.

#### 2.3.3. Efficacy requirements

Both MLV and inactivated vaccines have been evaluated for efficacy in vaccination – challenge studies. This involved respiratory challenge of a group of first-time vaccinated horses 4 weeks after primary immunisation, with the virulent

prototype Bucyrus strain of EAV. The level of protective immunity engendered by vaccination was assessed based on failure to produce clinical signs of EVA in the challenged horses or a significant reduction in the severity of disease compared to that observed in the nonvaccinated controls. The efficacy of vaccination in preventing establishment of the carrier state in vaccinated stallions was similarly evaluated.

#### 2.3.4. Duration of immunity

Detectable neutralising antibody titres to EAV should develop in the majority of horses within 1–2 weeks of vaccination with the MLV vaccine (Timoney & McCollum, 1993). Reported responses to primary vaccination have been variable in a couple of studies. In one stallion vaccination study, there was a rapid fall in antibody titres with a significant number of animals reverting to seronegativity 1–3 months after vaccination. On the other hand, other studies have been characterised by an excellent durable response, with persistence of high VN levels for at least 1–2 years. Revaccination with this vaccine results in an excellent anamnestic response, with the development of high antibody titres that remain relatively undiminished for several years (Timoney & McCollum, 1993).

Experimental studies have shown that most horses vaccinated with the inactivated vaccine develop low to moderate neutralising antibody titres to EAV by day 14 after the second vaccination. There is no published information on the duration of immunity conferred by this vaccine.

#### 2.3.5. Stability

The lyophilised MLV vaccine can be stored for at least 3–4 years at 2–7°C without loss in infectivity, provided it is kept in the dark. Infectivity is preserved for much longer periods if vaccine is frozen at –20°C or below. Once rehydrated, however, the vaccine should be used within 1 hour or else destroyed. The inactivated vaccine is stored as a liquid suspension at 2–8°C, with no loss of potency for at least 1 year, provided it is protected from light.

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