

EQUINE VIRAL ARTERITIS

SUMMARY

Equine viral arteritis (EVA) is a contagious viral disease of equids caused by equine arteritis virus (EAV), an RNA virus classified in the family Arteriviridae. Only one major serotype of the virus has been identified so far. Equine arteritis virus is found in horse populations in many countries worldwide. Although infrequently reported in the past, confirmed outbreaks of EVA appear to be on the increase.

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 based on virus isolation, detection of nucleic acid or viral antigen, or demonstration of a specific antibody response. Virus isolation should be attempted from appropriate clinical or post-mortem specimens in rabbit, equine, or monkey kidney cell culture. The identity of isolates of EAV should be confirmed by neutralisation test, reverse-transcription polymerase chain reaction (RT-PCR) assay, or by immunocytochemical methods, namely indirect immunofluorescence or avidin–biotin–peroxidase techniques.*

Detection and identification of EAV nucleic acid in suspect cases of the disease can also be attempted using the RT-PCR assay and appropriate viral-specific RNA primers.

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. In such cases, equine arteritis viral antigens may be visualised 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, none of which have been as extensively validated as the VN test though some appear to offer comparable specificity and close to equivalent sensitivity. The CF test is less sensitive than either procedure, but it can be used for diagnosing recent infection.*

Requirements for vaccines and diagnostic biologicals: *Two commercial tissue culture 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 cell cultures. It has been shown to be safe and protective for stallions and nonpregnant mares. Vaccination of foals under 6 weeks of age and of pregnant mares in the final 2 months of gestation is contraindicated. There is no evidence of back reversion to virulence of the vaccine virus following its use in the field over more than 20 years. 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

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* (10). Epizootic lymphangitis pinkeye, fièvre typhoïde and rotlaufseuche are some of the descriptive terms used in the past to refer to a disease that clinically very closely resembled 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 (63). The virus does not present a human health hazard (59). EAV is present in the horse population of many countries world-wide (59). 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 (2, 59).

While the majority of cases of acute infection with EAV are subclinical, certain strains of the virus can cause disease of varying severity (59). 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, 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 (37, 59, 62), and very rarely in otherwise healthy adult horses.

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, African horse sickness fever, cases of Hendra virus infection, Getah virus infection and toxicosis caused by hoary alyssum (*Berteroa incana*). After infection, EAV replicates in macrophages and circulating monocytes (18) and is shed in various secretions/excretions of acutely infected animals, in especially high concentration from the respiratory tract (42).

A variable percentage of acutely infected stallions later become long-term carriers in the reproductive tract and constant semen shedders of the virus (59, 60). 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 (59). Unequivocal evidence of the carrier state has only been found in stallions serologically positive for antibodies to the virus (60). 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.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

a) Virus isolation

Where an outbreak of EVA is suspected, or when attempting to confirm a case of subclinical EAV infection, virus isolation should be attempted from nasopharyngeal and conjunctival swabs, unclotted blood samples, and semen from stallions considered to be possible carriers of the virus (59). To optimise the chances of virus isolation, the relevant specimens should be obtained as soon as possible after the onset of fever in affected horses. There is evidence that heparin can inhibit the growth of EAV in rabbit kidney cells (RK-13 cell line) (1), and therefore, its use as an anticoagulant is contraindicated as it may interfere with isolation of the virus from whole blood. Acid citrate dextrose or ethylenediaminetetraacetic acid (EDTA) are the anticoagulants of choice to use in obtaining unclotted blood samples. Where EVA is suspected in cases of mortality in young foals or older animals, isolation of EAV 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 (42). 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 (59).

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 reverse-transcription polymerase chain reaction (RT-PCR) testing should be shipped either refrigerated or frozen in an insulated container to the laboratory,

preferably using an overnight delivery service. Unclothed blood samples must be transported refrigerated but not frozen. Where possible, specimens should be submitted to a laboratory with established competency to test for this infection.

Although reportedly not always successful in natural cases of EAV infection (43, 59), virus isolation should be attempted from clinical specimens or necropsied tissues using rabbit, equine or monkey kidney cell culture (43, 57, 59). Selected cell lines, e.g. RK-13 (ATCC CCL-37), LLC-MK₂ (ATCC CCL-7), and primary horse or rabbit kidney cell culture can be used, with RK-13 cells being the cell system of choice (57). 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 (49). Higher isolation rates have been obtained using 3–5-day-old 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. There is considerable evidence to indicate that primary isolation rates of EAV may be increased by using RK-13 cells of high passage history¹ (57).

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* (59, 60). The identity of isolates of EAV can be confirmed in a one-way neutralisation test, by standard RT-PCR or real-time RT-PCR assay (2, 12, 51, 53) or by an immunocytochemical method (36), namely indirect immunofluorescence (16) or the avidin–biotin–peroxidase (ABC) technique (36). A polyclonal rabbit antiserum has been used to identify EAV in infected cell cultures. Mouse monoclonal antibodies (MAbs) to the nucleocapsid protein (N) (2, 40) and major envelope glycoprotein (GP5) of EAV (2, 17) and a monospecific rabbit antiserum to the unglycosylated envelope protein (M) (2, 39, 40) 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 (2, 36).

- **Virus isolation from semen (a prescribed test for international trade)**

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 (59, 60). Stallions should first be blood tested using the virus neutralisation (VN) test or an appropriately validated enzyme-linked immunosorbent assay (ELISA) or other serological test procedure. Virus isolation should be attempted from the semen of stallions serologically positive (titre $\geq 1/4$) for antibodies to EAV that do not have a certified history of vaccination against EVA with confirmation that they were serologically negative (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 (59, 60). Immediately following collection, the semen should be refrigerated on crushed ice or on freezer packs for transport to the laboratory with a minimum of delay. Where there is likely to be a delay in submitting a specimen for testing, the semen can be frozen at or below -20°C for a short period before being dispatched to the laboratory. Freezing a sample has not been found to militate against isolation of EAV from the semen of a carrier stallion. In situations where it is not feasible to determine the carrier status of a stallion by virus isolation or RT-PCR procedures, the stallion can be test bred to two seronegative mares, which are checked for seroconversion to the virus 28 days after breeding (59).

- **Test procedure**

- i) On receipt in the laboratory, it should be noted whether each semen sample is frozen, chilled or at ambient temperature. Every sample should be checked to ensure that it contains the sperm-rich

¹ Such a line (RK-13-KY) is available from Dr P.J. Timoney, Maxwell H. Gluck Equine Research Center, University of Kentucky, Lexington, Kentucky 40546-0099, United States of America (E-mail address: ptimoney@uky.edu).

fraction of the ejaculate. This can be established by microscopic examination of a wet-mount preparation of a sample. Additionally, specimens of ejaculate should be visually inspected 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.

- ii) Although no longer considered an essential step, pretreatment of semen before inoculation into cell culture by short-term sonication (for three 15-second cycles); facilitates effective mixing and dispersion of a sample.
- iii) After removal of culture medium, 3–5-day-old confluent monolayer cultures of RK-13 cells, either in 25 cm² tissue culture flasks or multiwell plates, are inoculated with serial decimal dilutions (10⁻¹–10⁻³) of seminal plasma in tissue culture maintenance medium containing 2% fetal bovine serum and antibiotics. An inoculum of 1 ml per 25 cm² 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°C either in an aerobic incubator or an incubator containing a humidified atmosphere of 5% CO₂ 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°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 VN, immunofluorescence (16) or ABC technique, using a monospecific antiserum to EAV or MAbs to the structural proteins, N or GP5 of the virus (2, 18, 36, 37), or by standard RT-PCR (2, 6, 12, 27, 51, 53) or real-time RT-PCR assay (5, 38, 64).

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 cm² 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 virus isolate to be tested. Serial decimal dilutions (10⁻¹–10⁻⁵) of each virus are made in Eagles Minimal Essential Medium 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 3–5-day-old confluent monolayer cultures of RK-13 cells, either in 25 cm² 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% CO₂ 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 (34). Confirmation of the identity of an isolate is based on a reduction in plaque count of at least 10² 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 (59, 60). The occurrence of nonviral cytotoxicity or bacterial contamination of specimens is not considered significant problems when attempting isolation of this virus from stallion semen. Nonviral cytotoxicity, if observed, usually affects monolayers inoculated with the 10⁻¹ and, much less frequently, the 10⁻² 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 (25). The method

described involves the addition of polyethylene glycol to the 10^{-1} – 10^{-3} 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 (25). 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.

The presence of anti-EAV antibody activity in the seminal plasma of certain virus-shedding stallions has not been found to prevent detection of the carrier state in these animals.

b) Nucleic acid recognition methods

The standard two-step RT-PCR, single-step RT-PCR and real-time RT-PCR (rRT-PCR) assays are gaining greater acceptance and being more widely used as an alternative 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 swab filtrates, buffy coats, raw and extended semen and urine, and in post-mortem tissue samples (5, 6, 27, 55, 56, 64). Standard two-step RT-PCR, 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 (5, 6, 12, 27, 38, 50, 51, 53, 55, 56, 64, 66). 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 (6). 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 (5, 6, 9, 27, 38, 51, 53, 56, 66). Compared with traditional virus isolation, these RT-PCR-based assays are frequently more sensitive, less expensive 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 (5, 38). 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 because 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 (6). 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 many circumstances, use of the single-step RT-PCR or the one tube rRT-PCR assay would 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 (3, 4, 38, 64). 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 (5, 6, 38, 53, 56). 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']) (12) and/or [forward: 5'-CCT-GAG-ACA-CTG-AGT-CGC-GT-3' and reverse 5'-CCT-GAT-GCC-ACA-TGG-AAT-GA-3']) (27), ORF 6 ([forward: 5'-CTG-AGG-TAT-GGG-AGC-CAT-AG-3' and reverse: 5'-GCA-GCC-AAA-AGC-ACA-AAA-GC-3']) (51) and ORF 7 ([forward 5'-ATG-GCG-TCA-AGA-CGA-TCA-CG-3' and reverse 5'-AGA-ATA-TCC-ACG-TCT-TAC-GGC-3']) (53) 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 the RT-nPCR assay (6, 12, 27). While the RT-PCR has been found to be highly sensitive for viral nucleic acid detection in raw semen, there is evidence to that it is not of equivalent reliability when testing extended or cryopreserved semen of very low virus infectivity (66).

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 (5); 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 (64); 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 [38]).

In the absence of general agreement on a complete consensus or universal primer set for EAV, and since 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.

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 & 6 respectively) and the nucleocapsid (N) protein gene (ORF 7 [2, 8, 13, 54, 65]). The GP5 gene has been found to be most useful and reliable for this purpose (8, 54, 65). The relationships between strains demonstrated by nucleotide sequencing are a useful molecular epidemiological tool for tracing the origin of outbreaks of EVA (2, 4, 65).

c) Histopathological and immunohistochemical methods

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, particularly in the caecum, colon, spleen, associated lymphatic glands and adrenal cortex (16, 18, 33). The presence of a disseminated necrotising arteritis involving endothelial and medial cells of affected vessels is considered to be pathognomonic of EVA. The characteristic vascular lesions present in the mature animal are not, however, a prominent feature in many cases of EAV-related abortion (32).

EAV antigen can be identified in various tissues of EVA-affected animals either in the presence or absence of lesions (18). Antigen has been demonstrated in lung, heart, liver and spleen and the placenta of aborted fetuses (18, 56). 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 (16), or by the ABC technique using mouse MAbs to the GP5 (28) or N proteins of the virus (18, 37, 40, 56).

2. Serological tests

A variety of serological tests including neutralisation (microneutralisation [52] and plaque reduction [41] (VN)), the complement fixation (CF) test (22), the indirect fluorescent antibody test (16), the agar gel immunodiffusion (16), the ELISA (11, 14, 30, 31, 35, 48) and the fluorescent microsphere immunoassay (MIA) (Go, pers. comm.) have been used to detect antibody to EAV.

The test currently in widest international use to diagnose infection, carry out seroprevalence studies, and test horses for export, is a microneutralisation test in the presence of complement. It has also been used to screen fetal heart blood for the retrospective diagnosis of cases of EVA-related abortion (56). Apart from the VN test, the CF test has been used for diagnosing recent EAV infection as complement-fixing antibodies are relatively short-lived in duration (22). In contrast, neutralising antibody titres to EAV frequently persist for several years after natural infection (59). Although a number of ELISAs have been developed (11, 14, 30, 31, 35, 48), none has as yet been as extensively validated as the VN, though some appear to offer nearly comparable sensitivity and specificity (11, 30, 31, 48). 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.

Antiserum to unpurified EAV has been prepared in horses and in rabbits using conventional immunisation protocols. Also, mouse monoclonal and monospecific rabbit antibodies have been developed to the nucleocapsid protein (N) major envelope glycoprotein (GP5), and unglycosylated envelope protein (M) of EAV (7, 17, 28, 39, 40).

OIE Standard Sera for EAV are available² and these can facilitate international standardisation of the microneutralisation test and ELISA.

2 Available from Dr P.J. Timoney, Maxwell H. Gluck Equine Research Center, Dept of Veterinary Science, University of Kentucky, Lexington, Kentucky 40546-0099, United States of America; E-mail address: ptimoney@uky.edu.

Only one major serotype of EAV has been recognised so far (41, 59). This is represented by the prototype Bucyrus strain (ATCC VR 796). The reference virus used in the EAV VN test is the CVL-Bucyrus (Weybridge) strain. Virus stock is 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.

a) Virus neutralisation (a prescribed test for international trade)

The VN test is used to screen stallions for evidence of EAV infection and to determine whether there is a need to attempt virus detection in semen using cell culture or RT-PCR assay. It is also used for diagnostic purposes to confirm infection in suspect cases of EVA. The VN test procedure in current widest use is that developed by the National Veterinary Service Laboratories of the United States Department of Agriculture (52). 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 virus³ (20). Although originally derived from the prototype Bucyrus virus, the passage history of the CVL (Weybridge) strain is not fully documented. 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 (20, 21). 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.

• **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 to contain from 100 to 300 TCID₅₀ (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 control wells.
- 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% CO₂ in air.
- viii) A suspension of cells from 3–5-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% CO₂ 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 TCID₅₀ virus and that the titres of the positive serum controls are within 0.3 log₁₀ units of their predetermined titres.

A serum dilution is considered to be positive if there is an estimated 75% or preferably a 100% 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 (34). 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. Infrequently, sera will be

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encountered that cause 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 testing another serum sample from the animal in question or by retesting the toxic sample using microtitre plates with confluent monolayers of RK-13 cells that had been seeded the previous day. It has been reported that the toxicity of the serum can, in some cases, be reduced or eliminated if the sample is adsorbed with packed RK-13 cells prior to testing or by substituting rabbit in place of guinea-pig complement in the virus diluent. 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 dilutions and cause difficulties in interpretation of the test results (26, 47).

b) Enzyme-linked immunosorbent assay

A number of direct or indirect ELISAs have been developed for the detection of antibodies to EAV (11, 14, 30, 31, 35, 48). 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 (15). 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 (15). 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 (14, 17, 30). Most recently, an ovalbumin-conjugated synthetic peptide representing amino acids 81–106 of the GP5 protein has been used (48). 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 (11). 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 (31). 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 (30). 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 (7). 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 (14, 48). 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 (14). 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 (48). It is expected that an ELISA will soon be available, having very similar if not equivalent sensitivity and specificity to the VN test.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

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 prepared from virus that has been attenuated for horses by multiple serial transfers in equine and rabbit cell cultures (19, 41). 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 vaccine is commercially available in the USA and Canada. It has also been used in New Zealand, subject to ministerial controls, to aid in that country's EVA eradication programme.

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. The initial vaccination regimen involves two doses of vaccine administered intramuscularly 3–6 weeks apart. Booster vaccination at 6-month intervals is recommended by the manufacturer. The inactivated vaccine is licensed for commercial use in certain European countries, including Denmark, France, Germany, Hungary, Ireland, Sweden and the United Kingdom.

An additional inactivated vaccine against EVA has been developed in Japan for use should an outbreak of EVA occur in that country (24). 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 currently not commercially available, no details can be provided on its production.

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.

1. Seed management

a) Characteristics of the seed

Both MLV and inactivated commercial vaccines are derived from the prototype Bucyrus strain of EAV (ATCC VR 796). 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 (41, 59).

In the case of the MLV vaccine, the prototype virus 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) (19, 29, 41). The indications from the use of this vaccine are that the virus is safe and immunogenic between its 80th and 111th passage in primary rabbit kidney (19, 29, 41, 44, 45, 61).

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.

Suitable lots of master seed virus for each vaccine should be maintained in liquid nitrogen or its equivalent.

b) Method of culture

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.

c) Validation as a vaccine

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 (46). 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 (46, 52) 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. The newer technologies of PCR and antigen-capture ELISA may be used as adjuncts to virus isolation in screening for adventitious agents.

The MLV vaccine has been shown to be both safe and effective for use in stallions and nonpregnant mares (44, 45, 61). Although not recommended by the manufacturer for pregnant mares, especially in the last 2 months of gestation, the vaccine has been used to immunise pregnant mares in the face of high risk of natural exposure to EAV, with minimal, if any, reported adverse effects. Vaccination confers a high level of protective immunity that persists for at least several years (29, 41, 59). Based on experimental studies and extensive field use of the vaccine since 1985, there is no evidence of back reversion to virulence of the vaccine virus, nor of recombination of the vaccine virus with naturally occurring strains of EAV. Furthermore, there is no confirmed evidence that the attenuated strain of EAV in the current vaccine localises and sets up the carrier state in the reproductive tract of the vaccinated stallion (45, 59, 60, 61).

The commercial inactivated vaccine has been shown to be nonreactive and safe for use in healthy nonbreeding and breeding horses. Transient local reactions may be observed in less than 10% of horses

vaccinated with the inactivated vaccine. Limited field studies of this vaccine indicate that it is immunogenic, stimulating a satisfactory degree of immunity, the duration of which has yet to be reported.

Although there are no published reports on the efficacy of either commercial vaccine in preventing establishment of the carrier state in the stallion, an experimental aqueous formalin inactivated vaccine against EVA has been shown to prevent virus persistence in the reproductive tract of vaccinated stallions following subsequent challenge with EAV (23).

2. Method of manufacture

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.

3. In-process control

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.

4. Batch control

a) Sterility

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

b) Safety

In the case of both MLV and inactivated vaccines, each production lot of vaccine should be checked for extraneous bacterial, fungal and mycoplasmal contaminants. 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 (46). None of the inoculated horses should develop any clinical signs of disease other than mild pyrexia during the ensuing 2-week observation period. 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 (45, 59, 61). Limited shedding of vaccine virus by the respiratory route for at most 7 days may be demonstrated in the occasional vaccinated horse (61). There is no evidence of persistence of the vaccine virus in the reproductive tract after vaccination of stallions (45, 60, 61).

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.

c) 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 (46). 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 3×10^4 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.

d) 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 (44, 45, 58, 59, 61). 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 (61). 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 (58). Revaccination with this vaccine results in an excellent anamnestic response, with the development of high antibody titres that remain relatively undiminished for several years (59).

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.

e) 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 (29). 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.

f) Preservatives

The preservatives added to the MLV and inactivated vaccines are neomycin, polymyxin B and amphotericin B.

g) Precautions (hazards)

Pregnant mares should not be vaccinated with the MLV vaccine during the last 2 months of gestation, as there is a risk, albeit minimal, of fetal invasion by the vaccine virus. The possibility of a vaccinally induced anaphylactic reaction, though very rare, could result from the administration of either the MLV or inactivated vaccine. In the absence of appropriate safety data, the inactivated vaccine is currently not recommended for use in pregnant mares.

5. Tests on the final product

a) Safety

With the exception of the inactivated vaccine, which needs to be sterility tested a second time to ensure freedom from contamination, no further safety tests are required on the inactivated or MLV vaccines.

b) Potency

No potency tests additional to those conducted on each production lot of the MLV or inactivated vaccines are required on either final product.

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NB: There are OIE Reference Laboratories for Equine viral arteritis Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).