Equine Viral Arteritis

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SUMMARY

Equine viral arteritis (EVA) is a contagious viral disease of equids caused by equine arteritis virus (EAV), an RNA virus that is classified in the family Arteriviridae. The virus is found in horse populations in many countries worldwide. EVA cannot be differentiated clinically from a number of other respiratory and systemic equine diseases. While laboratory-confirmed outbreaks of EVA have been infrequently reported in the past, the incidence of the disease appears to be on the increase.

Although many cases of EAV infection are subclinical, certain virus strains can cause disease of varying severity 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 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 high percentage of infected stallions, but not in mares or nonbreeding horses.

Identification of the agent

Diagnosis of EAV infection is based on virus isolation or demonstration of a specific antibody response. Virus isolation should be attempted from nasopharyngeal and conjunctival swabs, unclotted blood samples, and semen from possible carrier stallions. In suspect cases of EVA-related mortality or abortion, isolation can be attempted from a wide range of tissues, especially lung, spleen and other lympho-reticular tissues, and from placental and fetal fluids and tissues. Isolation of EAV from clinical or post-mortem specimens should be attempted in rabbit, equine, or monkey kidney cell culture. The identity of isolates of EAV should be confirmed by neutralisation test or by immunocytochemical methods, namely indirect immunofluorescence or avidin–biotin–peroxidase techniques.

Where mortality is associated with a suspect 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.

Serological tests

A variety of serological tests, including neutralisation, complement fixation, indirect fluorescent antibody, agar gel immunodiffusion and the enzyme-linked immunosorbent assay (ELISA), have been used for the detection of antibody to EAV. The test currently in widest use is a complement-enhanced microneutralisation test. It is a sensitive and highly specific assay of proven value in diagnosing acute infection and in seroprevalence studies.

Status of Australia and New Zealand

Serological evidence of infection in both countries was found in 1988. Retrospective studies indicate that infection was present in Australia as early as 1975 and New Zealand in 1982. Infection is predominantly restricted to Standardbreds, and an industry-driven control program in New Zealand has led to a marked decline in the prevalence of infected horses. Clinical disease associated with infection has not been reported in either country.

Introduction

Serological evidence that the virus was present in Australia and New Zealand was found in 1988.[1] The source of infection was imported seropositive stallions from the USA, some of which were persistent shedders of virus in their semen. Retrospective serological studies have shown that the virus was present in Australia at least as far back as 1975 and New Zealand in 1982.

A serological study in Australia in 1990 found 4 of 50 (8%) Thoroughbred stallions and 37 of 51 (72.5%) Standardbred stallions positive.[2] In addition, EVA virus was isolated from 12 of 33 semen samples from recently imported stallions.

In New Zealand in 1990, 87 of 247 (36%) Standardbred stallions and 11 of 335 (3%) Thoroughbred stallions were seropositive. This represented 91% of Standardbred and 56% of Thoroughbred stallions.[3] The disease was made notifiable in 1989 and an industry-backed control scheme started soon after. The seroprevalence has decreased and currently 22% (72 of 315) of Standardbred stallions and 0.2% (13 of 634) of Thoroughbred stallions are seropositive. The number of chronic shedders has dropped from 20 to 3 and all are Standardbred. It seems hopeful the infection can be eradicated from New Zealand.

Aetiology

EVA is a contagious viral disease of equids caused by EVA virus, a positive-sense, single-stranded RNA virus, which is the prototype member of the genus *Arterivirus,* family Arteriviridae*,* order Nidovirales.[4] Epizootic lymphangitis-pinkeye, fievre typhoide and rotlaufseuche are some of the descriptive terms used in the past to refer to a disease that was very probably EVA. The natural host range of EVA virus would appear to be restricted to equids and the virus does not present a human health hazard.

Clinical disease due to EVA virus has not yet been reported in Australia or New Zealand. Recently, clinical EVA was reported for the first time in the United Kingdom[5] and the potential for an outbreak of disease is always present and should be considered if signs consistent with the disease are seen.

Epidemiology

The virus is maintained by stallions, which shed it continually in their semen.[6] About 30-50% of seropositive stallions are chronic virus shedders.

The output of EVA virus from persistently infected stallions is testosterone-dependent.[7] The carrier state has only been found in the stallion, not in the mare, gelding or sexually immature colt. Carrier stallions are seropositive so tests for serum antibody can be used to screen for potential carriers. Seropositive mares and geldings are those that have recovered from infection and are no longer shedders of virus. Acutely infected horses may transmit virus by aerosol.[5]

Clinical Signs

Whereas most cases of acute infection with EVA virus are subclinical, certain strains of the virus can cause disease of varying severity. Typical cases of EVA can present with any combination of the following clinical signs: fever (temperature up to 41°C), depression, anorexia, leukopenia, dependent oedema, especially of the limbs, scrotum and prepuce of the stallion, conjunctivitis, ocular discharge, supra- or peri-orbital oedema, rhinitis, nasal discharge, a local or generalised urticarial skin reaction, abortion and, rarely, a fulminating pneumonia 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 seen only in very young foals, especially those congenitally infected with the virus.

Rarely, abortion in pregnant mares may occur 2 to 3 weeks after the mare is infected. The foetus dies 2 to 3 days before it is aborted so is therefore not fresh when expelled, unlike abortions caused by equine herpesvirus type.[1]

Pathology

The lesions are referable to a severe generalised vasculitis. The virus replicates primarily in macrophages and endothelial cells with secondary sites being medial cells, mesothelium and epithelial cells of certain organs such as the adrenal cortex.[8] Oedema, congestion and haemorrhage of the subcutaneous tissues, lymph nodes and viscera of the peritoneal and pleural cavities are the most common gross findings. Further details have been reviewed.[8,9]

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, as prominent a feature in EVA-related abortions.

Diagnostic Tests

Diagnosis of EVA infection requires isolation and identification of virus from semen, tissues or swabs. More commonly it is based on sero-conversion using paired sera taken at a 2 – 4 week interval. In future, virus detection using the reverse-transcription polymerase chain reaction (RT-PCR) may become the method of choice.[10]

A range of tissues fixed in 10% buffered formalin should be taken for histopathological examination from aborted foetuses or horses that die from suspected EVA infection.

Virus isolation

In the case of a suspect outbreak of EVA or when attempting to confirm subclinical EVA virus 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. 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. Heparin is contraindicated as an anticoagulant when attempting virus isolation from blood as there is evidence that it inhibits the growth of EAV in RK-13 cells. Where EVA is suspected in cases of mortality in young foals or older animals, isolation of virus can be attempted from a variety of tissues, especially the lymph nodes associated with the alimentary tract and related organs, also the lungs, liver and spleen. In outbreaks involving abortion, placental and fetal fluids and a wide range of placental, lymphoreticular and other fetal tissues can be productive sources of virus.

Although not always successful in natural cases of EAV infection, virus isolation should be attempted from clinical specimens or tissues collected at necropsy by inoculation onto rabbit, equine or monkey kidney cell culture. Selected cell lines, for example, RK-13 (ATCC CCL-37), LLC-MK$_2$ (ATCC CCL-7), and African green monkey kidney (Vero) (ATCC CCL81) cells or primary horse or rabbit kidney cell culture can be used, with early passage RK-13 cells being the cell system of choice. There is evidence that higher passage levels of RK-13 cells can be less permissive for primary isolation, especially from semen. Several other factors have been shown to have an influence on the primary isolation of EVA virus from semen in RK-13 cells. Higher isolation rates have been obtained using 3- to 5-day-old monolayers, a large inoculum size in relation to the cell surface area in the inoculated flasks or multiwell plates, and the incorporation of carboxymethyl cellulose in the overlay medium.

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 fresh cell monolayers after 5 – 7 days. Most isolations are made in the first or second passage in cell culture.

There is considerable evidence that short- and long-term carrier stallions shed virus constantly in the semen but not in respiratory secretions or urine; nor has it been demonstrated in the buffy coat of the blood of such animals. 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 interval between collections or time of the year. Isolation should be carried out preferably on a 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 or disinfectants are used in the cleansing of the external genitalia of the stallion before collection. Samples should contain the sperm-rich fraction of the ejaculate with which virus is associated. The virus is not present in the pre-sperm fraction of semen. Immediately after 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 varying period of days or weeks 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 New Zealand and Australia, virus isolation is carried out in RK13 cells. Not all RK13 cell lines are suitable for EVA virus isolation. Two suitable RK13 cell lines are the ATCC RK13 cell line (CCL 37) and the University of Kentucky RK13 cell line (obtained from P. Timoney, Kentucky, USA). The latter is more sensitive and has been routinely used for EVA work in New Zealand. Both these cell lines are contaminated with bovine pestivirus. The role of this virus in the sensitivity of the cell line to EVA virus replication and production of a cytopathic effect is not certain. There is a suggestion that bovine pestivirus enhances the

production of CPE as pestivirus free RK13 cells were found to be not suitable for EVA work in the USA (A. Forman, personal communication). Laboratories using RK13 cells should be aware of the risks of cross-contamination of other susceptible cells with pestivirus and should handle these cells in a different laboratory or biohazard cabinet to that being used for other routine diagnostic work. It is necessary to use rapidly growing RK13 cells so cells should be passaged three times a week. Cells straight from liquid nitrogen have also been used routinely by some laboratories. Before freezing, the cells have been subcultured at weekly intervals and are then frozen, just before confluence, in small quantities suitable for the containers used for virus isolation on a single day. The other difference from normal cell culture isolation techniques is that it is necessary to include methyl cellulose in the maintenance media used after inoculation.

Isolates of EVA virus can be confirmed by neutralisation by monospecific antiserum, by immunochemical methods such as immuno-fluorescence and the avidin-biotin-peroxidase test, or by the RT-PCR.

A method for isolating EVA virus from semen is described in Appendix 1 and preparation of methyl cellulose overlay in Appendix 2.

Serological tests

Virus neutralisation test (VNT)

The microtitre VNT is the classical test used to measure serum antibodies.[11] It uses RK13 cells and the vaccine (Bucyrus) strain of virus. Complement is necessary if using strains of virus other than the modified Bucyrus strain of virus[12] and is included in the test described in this text. If complement is included when using the modified virus, titres are increased by about two-fold.

The test is read between 3 and 5 days, although often it is possible to read after only 2 days. Titres are calculated from the serum dilution, prior to addition of virus, at which there was no clear evidence of CPE in 50% of the wells. The test is described in detail in Appendix 3.

Enzyme-linked immunosorbant assay (ELISA)

A number of laboratories have developed the ELISA for serodiagnosis and serological surveys but it has not yet become widely accepted.[13]

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APPENDICES

Appendix 1

Isolation of EVA virus from semen

(a) Semen should be transported to the laboratory without delay on wet ice or frozen on dry ice. If dry ice is used, appropriate transport requirements should be checked (see UN1845).

(b) Sonicate approximately 10 mL of semen three times at 20 kHz for 15 sec intervals. Keep bottle containing semen in a beaker of crushed ice to prevent heating.

(c) Centrifuge at 1600 g for 10 min to clarify.

(d) Dilute 1.5 mL of seminal plasma with 13.5 mL of transport medium (TM) and ultracentrifuge at 30,000 rpm (63,000 g) for 90 min.

(e) Resuspend pellet in 1 mL of TM and make 10-fold dilutions in TM to 10^{-5}.

(f) Inoculate ATCC or Kentucky origin RK13 cells with 0.2 mL of the dilutions of resuspended pellet. Use one-day-old monolayers seeded at 1×10^5 cells/mL grown in 24 well plates. Start with undiluted pelleted specimen and inoculate 2 wells for each dilution.

(g) Adsorb for 1 h at 37°C. Wash once with 1 mL PBS. Then add an overlay of equal volumes of double strength Eagles MEM + 4% foetal calf serum and 1.5% methyl cellulose (see Appendix 2).

(h) Incubate in an atmosphere with 5% CO_2 at 37°C for 7 d, checking daily for CPE. A toxic effect is often present in the undiluted sample.

(i) If no CPE is detected, freeze and thaw cells/medium and pass onto fresh RK13 monolayers for further incubation for 7 days.

(j) Controls should include:
   - Cell control for each sample (inoculated with 0.2 mL of TM)
   - A known positive semen sample (previously tested and stored in small volumes at -70°C.
   - Stock virus diluted to 10^{-6}.

(k) A valid test requires:
   - No CPE in cell controls;
   - CPE present in wells inoculated with the positive semen sample; and
   - Virus titre of the stock virus to be within one log of that normally obtained with that stock.

Note well:

1. CPE caused by the stock virus is seen from day 2 onwards. CPE begins as focal plaques, which enlarge and coalesce to involve the whole cell monolayer. Early CPE can be difficult to differentiate from foci of piled up RK13 cells (overgrowth nodules).

2. A positive semen sample is recommended as a loss of cell sensitivity can occur which affects isolation of field strains of virus without significantly affecting the titre of the stock virus.

Appendix 2

Methyl cellulose overlay

To make up 400 mL of 1.5% overlay:

(a) Cool 100 mL of distilled water to 4°C.

(b) Heat 100 mL of distilled water to 90°C and add 3 g of methyl cellulose.

(c) Cool mixture (b) in an ice bucket and add chilled water (a).

(d) Dispense in 50 mL amounts in 100 mL bottles.

(e) Autoclave at 121°C for 20 min.

(f) Cool to 4°C shaking frequently. Store at 4°C.

(g) Add an equal volume of double-strength Eagles MEM with added bicarbonate and 4% foetal calf serum chilled to 4°C. Stir the mixture at 4°C for 2 hours.

(h) Dispense in 100 mL volumes and store at -20°C or make up fresh before use.

OR

Carboxymethyl cellulose (CMC) overlay

To make 3% CMC:

Add   3 g CMC (BDH, Product No: 27649

         100 mL H$_2$0

Stir at about 60°C until a clear viscous solution (usually overnight).

Dispense into appropriate volumes and autoclave at 115°C for 10 min.

Store at room temperature.

Appendix 3

Virus neutralisation test for EVA antibody

Materials:

Cells: Kentucky or ATCC RK13 cell line.

Media: 1. Eagles MEM without serum.

            2. Eagles MEM with 10% fresh guinea pig serum (virus diluent).

            3. Eagles MEM with 10% foetal calf serum (cell diluent). Add HEPES, NaHCO$_3$ and antibiotics as required.

Virus: EVA stock virus (vaccine or Bucyrus strain).

Positive control serum: Pooled positive field sera.

Negative control sera: Pooled negative field sera.

Procedure:

(a) Heat inactivate test sera at 56°C for 30 min.

(b) Using flat-bottomed, sterile, 96-well tissue-culture plates, dispense 50 µL of medium without serum to all wells except row A and cell control well on control plate (see (e) below).

(c) Dispense 50 µL of test serum into row A (serum control) and row B (for titration). Run each test serum in duplicate (Columns 1 and 2, 3 and 4, etc.).

(d) Titrate test sera, using a multihead pipettor, from row B to row H, in two-fold dilutions. Mix each dilution, thoroughly discarding 50 µL from row H when the dilution series is complete.

(e) Prepare a 'Control' plate on which:

   (i)   a known positive serum is titrated;

   (ii) a known negative serum is tested (at least in quadruplicate at a 1/4 dilution, but this may be titrated. Alternatively, a known negative serum can be included on one of the standard test plates and treated in the same manner as a 'test' serum);

   (iii) there is a cell control (containing only culture medium to a volume to replace all diluent, test serum and virus) (that is, 100 µL per well) to which only cells are added; and finally,

   (iv) a 'back' titration of the virus dilution used in the test (see i.).

(f) Dilute virus in medium containing 10% guinea pig complement to give 100 $TCID_{50}$/50 µL.

(g) Add 50 µL of medium plus complement to serum control and cell control wells.

(h) Add 50 µL of virus (100 $TCID_{50}$) to all wells except serum control wells (test plates) and cell control wells ('control' plate).

(i) Make a back titration of virus by making four, ten-fold dilutions of the test virus (100 $TCID_{50}$/50 µL) to give 10, 1, 0.1 and 0.01 $TCID_{50}$ per 50 µL. These dilutions are prepared in medium containing guinea pig complement and 50 µL of each dilution is inoculated into each of 8 wells on the 'control' plate.

(j) Incubate the covered plate in 5% CO_2 at 37°C for 1 h.

(k) Add 50 µL cells to all wells at a seeding rate of 3 x 10^5 cells per mL (1.5 x 10^4/well). If frozen cell stocks are used (straight from liquid nitrogen), the cells should be prepared in a manner to ensure that there is a 'working' stock (for use in test plates) at a consistent passage level. This is best achieved by producing these 'working' stocks from a master seed system to achieve a consistent 'end use' passage level. Generally, in producing a working stock, cells are passaged (approximately 1:6 split) at weekly intervals and frozen down at the preferred passage level when about 90% confluent (usually about 5 d growth). These cells are stored in quantities to seed a number of plates consistent with routine use on a single day (for example, one ampoule to three plates). Test plates are seeded with cells at a rate of 10^4 cells/well.

(l) Incubate in 5% CO_2 at 37°C for 3 to 5 d.

Interpretation

A serum dilution is considered positive if there is an estimated 75% or greater reduction in the amount of viral CPE in the serum test wells when compared with the CPE present in the wells of lowest virus dilution wells. Partial neutralisation may be observed over a range of dilutions so the highest dilution with at least 75% reduction in CPE is taken as the end point titre.

For the test to be valid:

- The cell controls on the control plate should contain a confluent monolayer of healthy cells.

- The virus titre employed in the test should not vary by more than +/- 0.3 log^{10} (that is, 1.7 - 2.3 log^{10}/50µL). If the virus titre is low, negative results may be accepted, but positives should be retested. When the virus titre is high, positive results will give a guide to titre, but all negatives should be retested.

- The negative serum control should show CPE in all wells.

- The positive serum control should not vary more than one dilution from its known titre in any one test.

- The test serum controls will indicate when a serum is toxic to the cells. If toxicity is encountered, a result cannot usually be reported for that serum.

- A titre of 1:4 or greater is considered to be positive.

General

- The VNT can be difficult to standardise due to fluctuations of the titre of the virus. Titres may vary by 2 or more logs depending on the growth rate of the RK13 cells. Cells need to be passaged 3 times a week to have them in the rapid growth phase. Cells direct from liquid nitrogen can be used and should be frozen in small volumes sufficient for several microplates – this will help minimise test variation.

- The RK13 cell concentration used in the test must be about 3 x10^5/mL for optimal monolayer development.

- Guinea pig complement must be fresh from guinea pig serum and stored at -70°C. Freeze-dried USA product is toxic.

- It is preferable to have the same technician doing EVA VNTs all the time.

- The modified live vaccine strain of EVA virus (Bucyrus) should be used. It will store 1 – 2 years at -70°C, but once the titre begins to fall it will fall rapidly. Freeze-dried stock is preferable.

- If the media becomes too alkaline (purple), replication of EVA virus is affected. This should not be a problem if plates are incubated in a well-maintained and calibrated humidified CO_2 incubator.

Appendix 4

Guinea pig complement production

(a) Anaesthetise guinea pigs with halothane or ether.

(b) Once anaesthetised, wash chest with 70% alcohol and iodine and heart puncture with a 22G x 1 inch needle. Using plain vacutainers withdraw 3 x 10 mL of blood per guinea pig. Euthanase the animals with barbiturate.

(c) Allow the blood to clot at room temperature for 2 h.

(d) Spin down the serum after aseptically removing clots.

(e) Pool all serum; dispense into 2 mL volumes and freeze at -70°C.

(f) From 8 guinea pigs it should be possible to get 50 to 70 mL of complement (serum).

(g) Check the complement in a test before using a new batch.