CHAPTER 3.5.9.

EQUINE RHINOPNEUMONITIS (INFECTION WITH EQUID HERPESVIRUS-1 AND -4)

SUMMARY

Equine rhinopneumonitis (ER) is a collective term for any one of several contagious, clinical disease entities of equids that may occur as a result of infection by either of two closely related herpesviruses, equid herpesvirus-1 and -4 (EHV-1 and EHV-4). Infection with EHV-1 is listed by the OIE. EHV-1 and EHV-4 are endemic in most domestic equine populations worldwide.

Primary infection by either EHV-1 or EHV-4 is characterised by upper respiratory tract disease of varying severity that is related to the age and immunological status of the infected animal. EHV-1 also causes the more serious complications of abortion, perinatal foal death, or paralytic neurological disease (equine herpesvirus myeloencephalopathy). EHV-4 has been associated with sporadic cases of abortion, but not the large outbreaks associated with EHV-1. Like other herpesviruses, EHV-1 and 4 induce long-lasting latent infections and can be reactivated following stress or pregnancy. Most horses are likely to be re-infected multiple times during their lifetime, often mildly or subclinically. Detection of viral DNA or anti-EHV antibodies should therefore be interpreted with care.

***Identification of the agent:** The standard method of identification of EHV-1 and EHV-4 from appropriate clinical or necropsy material is by polymerase chain reaction (PCR), followed by laboratory isolation of the virus in cell culture. Positive identification of viral isolates as EHV-1 or EHV-4 can be achieved by type-specific PCR. Viruses can be isolated in equine cell culture from nasal or nasopharyngeal swab extracts taken from horses during the febrile stage of respiratory tract infection, from the placenta and liver, lung, spleen, or thymus of aborted fetuses and early foal deaths, and from the leukocyte fraction of the blood of animals with acute EHV-1 infection. Unlike EHV-4, EHV-1 will also grow in various non-equine cell types such as the RK-13 cell line and this property can be used to distinguish between the two viruses.*

A rapid presumptive diagnosis of abortion induced by EHV-1 or (infrequently) EHV-4 can be achieved by direct immunofluorescent detection of viral antigen in cryostat sections of placenta and tissues from aborted fetuses, using a conjugated polyclonal antiserum.

Post-mortem demonstration of histopathological lesions of EHV-1 in placenta and tissues from aborted fetuses, cases of perinatal foal death or in the central nervous system of neurologically affected animals complements the laboratory diagnosis.

***Serological tests:** Most horses possess some level of antibody to EHV-1/4, the demonstration of specific antibody in the serum collected from a single blood sample is therefore not confirmation of a positive diagnosis of recent infection. Paired, acute and convalescent sera from animals suspected of being infected with EHV-1 or EHV-4 should be tested for a four-fold or greater rise in virus-specific antibody titre by either virus neutralisation (VN) or complement fixation (CF)* tests. *Neither of these assays is type-specific but both have proven useful for diagnostic purposes especially since the CF antibody response to recent infection is relatively short-lived. Limited use has also been made of a type-specific enzyme-linked immunosorbent assay (Crabb* et al., *1995; Hartley* et al., *2005).*

***Requirements for vaccines:** Both live attenuated and inactivated viral vaccines are available for use in assisting in the control of EHV-1/4. Vaccination is helpful in reducing the severity of respiratory infection in young horses and the incidence of abortion in mares, however current vaccines are not licenced to protect against neurological disease. Vaccination should not be* *considered a substitute for sound management practices known to reduce the risk of infection. Revaccination at frequent intervals is recommended in the case of each of the products, as the duration of vaccine-induced immunity is relatively short.*

Standards for production and licensing of both attenuated and inactivated EHV-1/4 vaccines are established by appropriate veterinary regulatory agencies in the countries of vaccine manufacture and use. A single set of internationally recognised standards for EHV vaccines is not available. In each case, however, vaccine production is based on the system of a detailed outline of production employing a well characterised cell line and a master seed lot of vaccine virus that has been validated with respect to virus identity, safety, virological purity, immunogenicity and the absence of extraneous microbial agents.

A. INTRODUCTION

Equine rhinopneumonitis (ER) is a historically-derived term that describes a constellation of several disease entities of horses that may include respiratory disease, abortion, neonatal foal pneumonitis, or myeloencephalopathy (Allen & Bryans, 1986; Allen *et al*., 1999; Bryans & Allen, 1988; Crabb & Studdert, 1995). The disease has been recognised for over 60 years as a threat to the international horse industry, and is caused by either of two members of the *Herpesviridae* family, equid herpesvirus-1 and -4 (EHV-1 and EHV-4). EHV-1 and EHV-4 are closely related alphaherpesviruses of horses with nucleotide sequence identity within individual homologous genes ranging from 55% to 84%, and amino acid sequence identity from 55% to 96% (Telford *et al*., 1992; 1998). The two herpesviruses are enzootic in all countries in which large populations of horses are maintained as part of the cultural tradition or agricultural economy. There is no recorded evidence that the two herpesviruses of ER pose any health risks to humans working with the agents. Infection with EHV-1 is listed by the OIE.

Viral transmission to cohort animals occurs by inhalation of aerosols of virus-laden respiratory secretions. Morbidity tends to be highest in young horses sharing the same air space. Aborted tissues and placental fluids from infected mares can contain extremely high levels of live virus and represent a major source of infection. Extensive use of vaccines has not eliminated EHV infections, and the world-wide annual financial impact from these equine pathogens is immense.

In horses under 3 years of age, clinical ER usually takes the form of an acute, febrile respiratory illness that spreads rapidly through the group of animals. The viruses infect and multiply in epithelial cells of the respiratory mucosa. Signs of infection become apparent 2–8 days after exposure to virus, and are characterised by fever, inappetence, depression, and nasal discharge. The severity of respiratory disease varies with the age of the horse and the level of immunity resulting from previous vaccination or natural exposure. Fever and complications are more likely with EHV-1 than EHV-4. Subclinical infections with EHV-1/4 are common, even in young animals. Although mortality from uncomplicated ER is rare and complete recovery within 1–2 weeks is the normal outcome, respiratory infection is a frequent and significant cause of interrupted schedules among horses assembled for training, racing, or other equestrian events. Fully protective immunity resulting from infection is of short duration, and convalescent animals are susceptible to reinfection by EHV-1/4 after several months. Although reinfections by the two herpesviruses cause less severe or clinically inapparent respiratory disease, the risks of subsequent abortion or neurological disease remain. Like other herpesviruses, EHV-1/4 cause long-lasting latent infections and latently infected horses represent a potential infection risk for other horses. Virus can be reactivated as a result of stress or pregnancy. The greatest clinical threats to individual breeding, racing, or pleasure horse operations posed by ER are the potential abortigenic and neurological *sequelae* of EHV-1 respiratory infection.

Neurological disease, also known as equine herpesvirus myeloencephalopathy, remains an infrequent but serious complication of EHV-1 infection. A single mutation in the DNA polymerase gene (ORF30) has been associated with increased risk of neurological disease, however strains without this change can also cause paralysis (Nugent *et al.,* 2006; Goodman *et al*., 2007). Strain typing techniques have been employed to identify viruses carrying the neuropathic marker, and it can be helpful to be aware of an increased risk of neurological complications. However, for practical purposes strain-typing is not relevant for agent identification, or international trade. Strain typing may be beneficial for implementation of biosecurity measures in the management of outbreaks of equine herpesvirus myeloencephalopathy.

B. DIAGNOSTIC TECHNIQUES

Both EHV-1 and EHV-4 have the potential to be highly contagious viruses and the former can cause explosive outbreaks of abortion or neurological disease. Rapid diagnostic methods are therefore useful for managing the disease. Polymerase chain reaction (PCR) assays are widely used by diagnostic laboratories and are both rapid and sensitive. Real-time PCR assays that allow simultaneous testing for EHV-1 and EHV-4 and quantification of viral load have been developed. Virus isolation can also be useful, particularly for the detection of viraemia. This is also true of EHV-1 associated abortions and neonatal foal deaths, when the high level of virus in the tissues usually produces a cytopathic effect in 1–3 days. Immunohistochemical or immunofluorescent approaches can be extremely useful for rapid diagnosis of EHV-induced abortion from fresh or embedded tissue and are relatively straightforward. Several other techniques based on enzyme-linked immunosorbent assay (ELISA) or nucleic acid hybridisation probes have also been described, however their use is often restricted to specialised laboratories and they are not included here.

Table 1. *Test methods available for the diagnosis of equine rhinopneumonitis and their purpose*

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection - surveillance	Immune status in individual animals or populations post-vaccination
Agent identification[1]						
Virus isolation	–	+++	–	+++	–	–
PCR	–	+++	–	+++	–	–
Detection of immune response						
VN	+	+	+	+++	+++	+++
ELISA	+	+	+	++	+++	+
CFT	–	–	–	+++	–	–

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.
Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
PCR = polymerase chain reaction; VN = virus neutralisation;
ELISA = enzyme-linked immunosorbent assay; CFT = complement fixation test.

1. Identification of the agent

1.1. Collection and preparation of specimens

Nasal/nasopharyngeal swabs: swab extract can be used for DNA extraction and subsequent virus detection by PCR using one of a variety of published techniques or commercially available kits (see below). Virus isolation can also be attempted from the swab extracts. To increase the chances of isolating live virus, swabs are best obtained from horses during the very early, febrile stages of the respiratory disease, and are collected via the nares by sampling the area with a swab of an appropriate size and length for horses. After collection, the swab should be removed and transported immediately to the virology laboratory in 3 ml of cold (not frozen) virus transport medium (e.g. PBS or serum-free MEM [minimal essential medium] with antibiotics). Virus infectivity can be prolonged by the addition of bovine serum albumin, fetal calf serum or gelatine to 0.1% (w/v).

Tissue samples: total DNA can be extracted using a number of commercially available kits and used in PCR to detect viral DNA (described below in Section B.1.2.1). Virus isolation from placenta and fetal

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

tissues from suspect cases of EHV-1 abortion is most successful when performed on aseptically collected samples of placenta, liver, lung, thymus and spleen. Virus may be isolated from post-mortem cases of EHV-1 neurological disease by culture of samples of brain and spinal cord but such attempts to isolate virus are often unsuccessful; however, they may be useful for PCR testing and pathological examination. Tissue samples should be transported to the laboratory and held at 4°C until inoculated into tissue culture. Samples that cannot be processed within a few hours should be stored at –70°C.

Blood: for virus isolation from blood leukocytes, collect a 20 ml sample of blood, using an aseptic technique in citrate, heparin or EDTA [ethylene diamine tetra-acetic acid] anticoagulant. EDTA is the preferred anticoagulant for PCR testing. The samples should be transported without delay to the laboratory on ice, but not frozen.

1.2. Virus detection by polymerase chain reaction

PCR has become the primary diagnostic method for the detection of EHV-1 and EHV-4 in clinical specimens, paraffin-embedded archival tissue, or inoculated cell cultures (Borchers & Slater, 1993; Lawrence *et al*., 1994; O'Keefe *et al*., 1994; Varrasso *et al*., 2001). A variety of type-specific PCR primers have been designed to distinguish between the presence of EHV-1 and EHV-4. The correlation between PCR and virus isolation techniques for diagnosis of EHV-1 or EHV-4 is high (Varrasso *et al*., 2001). Diagnosis by PCR is rapid, sensitive, and does not depend on the presence of infectious virus in the clinical sample.

For diagnosis of active infection by EHV, PCR methods are most reliable with tissue samples from aborted fetuses and placental tissue or from nasopharyngeal swabs of foals and yearlings. They are particularly useful in explosive outbreaks of abortion, respiratory or neurological disease in which a rapid identification of the virus is critical for guiding management strategies, including movement restrictions. PCR examination of spinal cord and brain tissue, as well as peripheral blood mononuclear cells (PBMC), are important in seeking a diagnosis on a horse with neurological signs.

Several PCR assays have been published. A nested PCR procedure can be used to distinguish between EHV-1 and EHV-4. A sensitive protocol suitable for clinical or pathological specimens (nasal secretions, blood leukocytes, brain and spinal cord, fetal tissues, etc.) has been described by Borchers & Slater (1993). However, nested PCR methods have a high risk of laboratory cross-contamination, and sensitive rapid one-step PCR tests to detect EHV-1 and EHV-4 (e.g. Lawrence *et al.,* 1994) are preferred. The OIE Reference Laboratories use quantitative real-time PCR assays such as those targeting heterologous sequences of major glycoprotein genes to distinguish between EHV-1 and EHV-4. A multiplex real-time PCR targeting glycoprotein B gene of EHV-1 and EHV-4 was described by Diallo *et al.* (2007). PCR protocols have been developed that can differentiate between EHV-1 strains carrying the ORF30 neuropathogenic marker, using both restriction enzyme digestion of PCR products (Fritsche & Borchers, 2011) or by quantitative real-time PCR (Allen *et al,* 2007, Smith *et al.*, 2012). Methods have also been developed to type strains for epidemiological purposes, based on the ORF68 gene (Nugent *et al.,* 2006). The OIE reference laboratories employ in-house methods for strain typing, however these protocols have not yet been validated between different laboratories at an international level.

Real-time (or quantitative) PCR has become the method of choice for many diagnostic tests and provides rapid and sensitive detection of viral DNA. Real-time PCR assays have been described for EHV-1 and EHV-4. The real-time PCR test outlined below has been validated to ISO 17025 at the UK OIE Reference Laboratory and is designed for use in a 96-well format. This can be readily combined with automatic nucleic acid extraction methods. This multiplex assay amplifies viral DNA sequences specific to either EHV-1 or EHV-4 in equine tissue samples, nasal swabs, or respiratory washes. It has not been validated for use with whole blood or buffy coat. The target region for amplification of each virus is in a conserved type-specific area of the gene for glycoprotein B (gB) for EHV-1 and ORF17 (encoding UL43) for EHV-4. Discrimination between EHV-1 and EHV-4 is carried out by the incorporation of type-specific dual labelled probes. The method uses in-house designed primers and probes, based on methods published by Hussey *et al.* (2006) and Lawrence *et al.* (1994). To establish such a real-time PCR assay for diagnostic purposes, validation against blinded samples is required. Sensitivity and specificity for the assay should be determined against each target. Support for development of assays and appropriate sample panels can be obtained from the OIE Reference Laboratories.

Alternative validated protocols may be used, with appropriate optimisation of thermocycler times and temperatures, for example the methods of Diallo *et al.* (2006; 2007), Allen (2007).

1.2.1. Test protocol

i) Suitable specimens

Equine post-mortem tissues from newborn and adult animals or equine fetal tissue from abortions (tissues containing lung, liver, spleen, thymus, adrenal gland and placental tissues) can be used. For respiratory samples, equine nasopharyngeal swabs or deep nasal swabs (submitted in a suitable viral transport medium), tracheal wash (TW) or broncho-alveolar lavage (BAL) are all suitable. DNA should be extracted using an appropriate kit or robotic system.

ii) Primers and probes

EHV 1 Forward: GGG-GTT-CTT-AAT-TGC-ATT-CAG-ACC

EHV 1 Reverse: GTA-GGT-GCG-GTT-AGA-TCT-CAC-AAG

EHV 4 Forward: TAG-CAA-ACA-CCC-ACT-AAT-AAT-AGC-AAG

EHV 4 Reverse: GCT-CAA-ATC-TCT-TTA-TTT-TAT-GTC-ATA-TGC

EHV1gB/probe: {FAM}TCT-CCA-ACG-AAC-TCG-CCA-GGC-TGT-ACC{BHQ1}

EHV4ORF17/probe: {JOE}CGG-AAC-AGG-AAC-TCA-CTT-CAG-AGC-CAG-C{BHQ1}

iii) Real-time PCR standards

A DNA standard curve should be used to quantify the levels of viral DNA, comprising at least four standards containing EHV-1 and EHV-4 target DNA at known concentrations. All standards should be diluted in 1 ng/ml Polyinosinic–polycytidylic acid (PolyI/C) to stabilise the DNA in solution. These should be stored at –20°C and not subjected to multiple rounds of freeze–thaw. Suitable plasmids are available on request from the OIE Reference Laboratory in the UK.

iv) Test procedure

Due to the extreme sensitivity of PCR based tests, it is vital to eliminate all possible sources of nucleic acid contamination. All equipment and reagents must be of molecular biology/PCR grade and be guaranteed free from contaminating nucleic acids, nucleases, or other interfering enzymes.

Reactions should be prepared with appropriate PCR master mix kits. Reactions and collection of data are carried out in a real-time thermocycler using conditions that are optimised for that machine. The amount of viral DNA in each sample can be quantified against known DNA standards, however suitable positive and negative controls should also be included on each run: water as a non-template control, buffer that has been subjected to the sample extraction method (negative extraction control) and EHV-1 and EHV-4 virus as positive extraction controls. To ensure the ongoing quality of the assay, the cycle threshold (Ct) of a known low copy standard (e.g. 100 copies) should be recorded for each run and monitored regularly.

1.3. Virus isolation

For efficient primary isolation of EHV-4 from horses with respiratory disease, equine-derived cell cultures must be used. Both EHV-1 and EHV-4 may be isolated from nasopharyngeal samples using primary equine fetal kidney cells or equine fibroblasts derived from dermal (E-Derm) or lung tissue. EHV-1 can be isolated on other cell types, as will be discussed later. The nasopharyngeal swab and its accompanying transport medium are transferred into the barrel of a sterile 10 ml syringe. Using the syringe plunger, the fluid is squeezed from the swab into a sterile tube. A portion of the expressed fluid can be filtered through a sterile, 0.45 µm membrane syringe filter unit into a second sterile tube if heavy bacterial contamination is expected, but this may also lower virus titre. Recently prepared cell monolayers in tissue culture flasks are inoculated with the filtered, as well as the unfiltered, nasopharyngeal swab extract. Cell monolayers in multiwell plates incubated in a 5% CO_2 environment may also be used. Virus is allowed to attach by incubating the inoculated monolayers at 37°C. Monolayers of uninoculated control cells should be incubated in parallel.

At the end of the attachment period, the inocula are removed and the monolayers are rinsed twice with phosphate buffered saline (PBS) to remove virus-neutralising antibody that may be present in the nasopharyngeal secretions. After addition of supplemented maintenance medium (MEM containing 2% fetal calf serum [FCS] and twice the standard concentrations of antibiotics/antifungals [penicillin, streptomycin, gentamicin, and amphotericin B]), the flasks are incubated at 37°C. The use of positive control virus samples to validate the isolation procedure carries the risk that this may lead to eventual contamination of diagnostic specimens. This risk can be minimised by using routine precautions and good laboratory technique, including the use of biosafety cabinets, inoculating positive controls after the diagnostic specimens, decontaminating the surfaces in the hood while the inoculum is adsorbing and using a positive control of relatively low titre. Inoculated flasks should be inspected daily by microscopy for the appearance of characteristic herpesvirus cytopathic effect (CPE) (focal rounding, increase in refractility, and detachment of cells). Cultures exhibiting no evidence of viral CPE after 1 week of incubation should be blind-passaged into freshly prepared monolayers of cells, using small aliquots of both media and cells as the inoculum. Further blind passage is usually not productive.

Tissue samples: A number of cell types may be used for isolation of EHV-1 (e.g. rabbit kidney [RK-13 (AATC–CCL37)], baby hamster kidney [BHK-21], Madin–Darby bovine kidney [MDBK], pig kidney [PK-15], etc.). It can be useful to inoculate samples into both non-equine and equine cells in parallel to distinguish between EHV-1 and EHV-4, since EHV-4 can cause sporadic cases of abortion. Around 10% (w/v) pooled tissue homogenates of liver, lung, thymus, and spleen (from aborted fetuses) or of central nervous system tissue (from cases of neurological disease) are used for virus isolation. These are prepared by first mincing small samples of tissue into 1 mm cubes in a sterile Petri dish with dissecting scissors, followed by macerating the tissue cubes further in serum-free culture medium with antibiotics using a homogeniser or mechanical tissue grinder. After centrifugation at 1200 ***g*** for 10 minutes, the supernatant is removed and 0.5 ml is inoculated into duplicate cell monolayers in tissue culture flasks. Following incubation of the inoculated cells at 37°C for 1.5–2 hours, the inocula are removed and the monolayers are rinsed twice with PBS or maintenance medium. After addition of 5 ml of supplemented maintenance medium, the flasks are incubated at 37°C for up to 1 week or until viral CPE is observed. Cultures exhibiting no evidence of viral CPE after 1 week of incubation should be passaged a second time into freshly prepared monolayers of cells, using small aliquots of both media and cells as the inoculum.

Blood samples: EHV-1 and, infrequently, EHV-4 can be isolated from PBMC. Buffy coats may be prepared from unclotted blood by centrifugation at 600 ***g*** for 15 minutes, and the buffy coat is taken after the plasma has been carefully removed. The buffy coat is then layered onto a PBMC separating solution (Ficoll; density 1077 g/ml, commercially available) and centrifuged at 400 ***g*** for 20 minutes. The PBMC interface (without most granulocytes) is washed twice in PBS (300 ***g*** for 10 minutes) and resuspended in 1 ml of MEM containing 2% FCS. As a quicker alternative method, PBMC may be collected by centrifugation directly from plasma. An aliquot of the rinsed cell suspension is added to each of the duplicate monolayers of equine fibroblast, equine fetal or RK-13 cell monolayers in 25 cm^2 flasks containing 8–10 ml freshly added maintenance medium. The flasks are incubated at 37°C for 7 days; either with or without removal of the inoculum. If PBMCs are not removed prior to incubation, CPE may be difficult to detect in the presence of the massive inoculum of leukocytes: each flask of cells is freeze–thawed after 7 days of incubation and the contents centrifuged at 300 ***g*** for 10 minutes. Finally, 0.5 ml of the cell-free, culture medium supernatant is transferred to freshly made cell monolayers that are just subconfluent. These are incubated and observed for viral CPE for at least 5–6 days. Again, samples exhibiting no evidence of viral CPE after 1 week of incubation should be passaged a second time before discarding as negative.

Virus identity may be confirmed by PCR or by immunofluorescence with specific antisera. Virus isolates from positive cultures should be submitted to an OIE reference laboratory to maintain a geographically diverse archive. Further strain characterisation for surveillance purposes or detection of the neurological marker can be provided at some laboratories.

1.4. Virus detection by direct immunofluorescence

Direct immunofluorescent detection of EHV antigens in samples of post-mortem tissues collected from aborted equine fetuses and the placenta provides a rapid preliminary diagnosis of herpesvirus abortion (Gunn, 1992). The diagnostic reliability of this technique approaches that of virus isolation attempts from the same tissues.

In the United States of America (USA), potent polyclonal antiserum to EHV-1, prepared in swine and conjugated with FITC, is available to veterinary diagnostic laboratories for this purpose from the National Veterinary Services Laboratories of the United States Department of Agriculture (USDA). The antiserum cross-reacts with EHV-4 and hence is not useful for serotyping, however virus typing can be conducted on any virus positive specimens by PCR.

Freshly dissected samples (5 × 5 mm pieces) of fetal tissue (lung, liver, thymus, and spleen) are frozen, sectioned on a cryostat at –20°C, mounted on to microscope slides, and fixed with 100% acetone. After air-drying, the sections are incubated at 37°C in a humid atmosphere for 30 minutes with an appropriate dilution of the conjugated swine antibody to EHV-1. Unreacted antibody is removed by two washes in PBS, and the tissue sections are then covered with aqueous mounting medium and a cover-slip, and examined for fluorescent cells indicating the presence of EHV antigen. Each test should include a positive and negative control consisting of sections from known EHV-1 infected and uninfected fetal tissue.

1.5. Virus detection by immunoperoxidase staining

Immunohistochemical (IH) staining methods, such as immunoperoxidase, have been developed for detecting EHV-1 antigen in fixed tissues of aborted equine fetuses, placental tissues or neurologically affected horses (Schultheiss *et al*., 1993; Whitwell *et al*., 1992). Such techniques can be used as an alternative to immunofluorescence described above and can also be readily applied to archival tissue samples. Immunohistochemical staining for EHV-1 is particularly useful for the simultaneous evaluation of morphological lesions and the identification of the virus. Immunoperoxidase staining for EHV-1/4 may also be carried out on infected cell monolayers (van Maanen *et al*., 2000). Adequate controls must be included with each immunoperoxidase test run for evaluation of both the method specificity and antibody specificity. In one OIE reference laboratory, this method is used routinely for frozen or fixed tissue, using rabbit polyclonal sera raised against EHV-1. This staining method is not type-specific and therefore needs to be combined with virus isolation or PCR to discriminate between EHV-1 and EHV-4, however it provides a useful method for rapid diagnosis of EHV-induced abortion.

1.6. Histopathology

Histopathological examination of sections of fixed placenta and lung, liver, spleen, adrenal and thymus from aborted fetuses and brain and spinal cord from neurologically affected horses should be carried out. In aborted fetuses, eosinophilic intranuclear inclusion bodies present within bronchiolar epithelium or in cells at the periphery of areas of hepatic necrosis are consistent with a diagnosis of herpesvirus infection. The characteristic microscopic lesion associated with EHV-1 neuropathy is a degenerative thrombotic vasculitis of small blood vessels in the brain or spinal cord (perivascular cuffing and infiltration by inflammatory cells, endothelial proliferation and necrosis, and thrombus formation).

2. Serological tests

EHV-1 and EHV-4 are endemic in most parts of the world and seroprevalence is high, however serological testing of paired sera can be useful for diagnosis of ER in horses. A positive diagnosis is based on the demonstration of significant increases (four-fold or greater) in antibody titres in paired sera taken during the acute and convalescent stages of the disease. The results of tests performed on sera from a single collection date are, in most cases, impossible to interpret with any degree of confidence. The initial (acute phase) serum sample should be taken as soon as possible after the onset of clinical signs, and the second (convalescent phase) serum sample should be taken 2–4 weeks later.

'Acute phase' sera from mares after abortion or from horses with EHV-1 neurological disease may already contain maximal titres of EHV-1 antibody, with no increase in titres detectable in sera collected at later dates. In such cases, serological testing of paired serum samples from clinically unaffected cohort members of the herd may prove useful for retrospective diagnosis of ER within the herd.

Finally, the serological detection of antibodies to EHV-1 in heart or umbilical cord blood or other fluids of equine fetuses can be of diagnostic value in cases of abortion especially when the fetus is virologically negative. The EHV 1/4 nucleic acid may be identified from these tissues by PCR.

Serum antibody levels to EHV-1/4 may be determined by virus neutralisation (VN) (Thomson *et al*., 1976), complement fixation (CF) tests (Thomson *et al*., 1976) or ELISA (Crabb *et al*., 1995). There are no internationally recognised reagents or standardised techniques for performing any of the serological tests for detection of EHV-1/4 antibody; titre determinations on the same serum may differ from one laboratory to another. Furthermore, the CF and VN tests detect antibodies that are cross-reactive between EHV-1 and EHV-4. Nonetheless, the demonstration of a four-fold or greater rise in antibody titre to EHV-1 or EHV-4 during the course of a clinical illness provides serological confirmation of recent infection with one of the viruses.

The microneutralisation test is a widely used and sensitive serological assay for detecting EHV-1/4 antibody and will thus be described here.

2.1. Virus neutralisation test

This test is most commonly performed in flat-bottom 96-well microtitre plates (tissue culture grade) using a constant dose of virus and doubling dilutions of equine test sera. At least two replicate wells for each serum dilution are required. Serum-free MEM is used throughout as a diluent. Virus stocks of known titre are diluted just before use to contain 100 $TCID_{50}$ (50% tissue culture infective dose) in 25 µl. Monolayers of E-Derm or RK-13 cells are monodispersed with EDTA/trypsin and resuspended at a concentration of 5×10^5/ml. Note that RK-13 cells can be used with EHV-1 but do not show CPE with EHV-4. Antibody positive and negative control equine sera and controls for cell viability, virus infectivity, and test serum cytotoxicity, must be included in each assay. End-point VN titres of antibody are calculated by determining the reciprocal of the highest serum dilution that protects 100% of the cell monolayer from virus destruction in both of the replicate wells.

Serum toxicity may be encountered in samples from horses repeatedly vaccinated with a commercial vaccine prepared from EHV-1 grown up in RK-13 cells. This can give rise to difficulties in interpretation of test reactions at lower serum dilutions. The problem can be overcome using E-Derm or other non-rabbit kidney derived cell line.

2.1.1. Test procedure

A suitable test procedure is as follows:

i) Inactivate test and control sera for 30 minutes in a water bath at 56°C.

ii) Add 25 µl of serum-free MEM to all wells of the microtitre assay plates.

iii) Pipette 25 µl of each test serum into duplicate wells of both rows A and B of the plate. The first row serves as the serum toxicity control and the second row as the first dilution of the test. Make doubling dilutions of each serum starting with row B and proceeding to the bottom of the plate by sequential mixing and transfer of 25 µl to each subsequent row of wells. Six sera can be assayed in each plate.

iv) Add 25 µl of the appropriately diluted EHV-1 or EHV-4 virus stock to each well (100 $TCID_{50}$/well) except those of row A, which are the serum control wells for monitoring serum toxicity for the indicator cells. Note that the final serum dilutions, after addition of virus, run from 1/4 to1/256.

v) A separate control plate should include titration of both a negative and positive horse serum of known titre, cell control (no virus), virus control (no serum), and a virus titration to calculate the actual amount of virus used in the test.

vi) Incubate the plates for 1 hour at 37°C in 5% CO_2 atmosphere.

vii) Add 50 µl of the prepared E-Derm or RK-13 cell suspension (5×10^5 cells/ml) in MEM/10% FCS to each well.

viii) Incubate the plates for 4–5 days at 37°C in an atmosphere of 5% CO_2 in air.

ix) Examine the plates microscopically for CPE and record the results on a worksheet. Alternatively, the cell monolayers can be scored for CPE after fixing and staining as follows: after removal of the culture fluid, immerse the plates for 15 minutes in a solution containing 2 mg/ml crystal violet, 10% formalin, 45% methanol, and 45% water. Then, rinse the plates vigorously under a stream of running tap water.

x) Wells containing intact cell monolayers stain blue, while monolayers destroyed by virus do not stain. Verify that the cell control, positive serum control, and serum cytotoxicity control wells stain blue, that the virus control and negative serum control wells are not stained, and that the actual amount of virus added to each well is between $10^{1.5}$ and $10^{2.5}$ $TCID_{50}$. Wells are scored as positive for neutralisation of virus if 100% of the cell monolayer remains intact. The highest dilution of serum resulting in complete neutralisation of virus (no CPE) in both duplicate wells is the end-point titre for that serum.

xi) Calculate the neutralisation titre for each test serum, and compare acute and convalescent phase serum titres from each animal for a four-fold or greater increase.

C. REQUIREMENTS FOR VACCINES

1. Background

Both live attenuated and inactivated vaccines are available for use in horses as licensed, commercially prepared products for use in reducing the impact of disease in horses caused by EHV-1/4 infection. The products contain different permutations of EHV-1 and EHV-4 and some also include equine influenza virus.

Clinical experience has demonstrated that vaccination can be useful for reducing clinical signs of respiratory disease and incidence of abortion, however none of the vaccines protects against neurological disease. Multiple doses repeated annually, of each of the currently marketed ER vaccines are recommended by their respective manufacturers. Vaccination schedules vary with a particular vaccine.

The indications stated on the product label for use of several available vaccines for ER are either as a preventative of herpesvirus-associated respiratory disease, or as an aid in the prevention of abortion, or both. Only four vaccine products have met the regulatory requirements for claiming efficacy in providing protection from herpesvirus abortion as a result of successful vaccination and challenge experiments in pregnant mares. None of the vaccine products has been demonstrated to prevent the occurrence of neurological disease sometimes associated with EHV-1 infection.

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

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

The master seed virus (MSV) for ER vaccines must be prepared from strains of EHV-1 and/or EHV-4 that have been positively and unequivocally identified by both serological and genetic tests. Seed virus must be propagated in a cell line approved for equine vaccine production by the appropriate regulatory agency. A complete record of original source (including isolate number, location, year of isolation), passage history, medium used for propagation, etc., shall be kept for the master seed preparations of both the virus(es) and cell stock(s) intended for use in vaccine production.

2.1.1. Biological characteristics of the master seed

Permanently stored stocks of both MSV and master cell stock (MCS) used for vaccine production must be demonstrated to be pure, safe and, in the case of MSV, also immunogenic.

Generally, the fifth passage from the MSV and the twentieth passage from the MCS are the highest allowed for vaccine production. Results of all quality control tests on master seeds must be recorded and made a part of the licensee's permanent records.

2.1.2. Quality criteria

Tests for master seed purity include prescribed procedures that demonstrate the virus and cell seed stocks to be free from bacteria, fungi, mycoplasmas, and extraneous viruses. Special tests must be performed to confirm the absence of equine arteritis virus, equine infectious anaemia virus, equine influenza virus, equine herpesvirus-2, -3, and -5, equine rhinitis A and B viruses, the alphaviruses of equine encephalomyelitis, bovine viral diarrhoea virus (BVDV – common contaminant of bovine serum), and porcine parvovirus (PPV – potential contaminant of porcine trypsin). The purity check should also include the exclusion of the presence of EHV-1 from EHV-4 MSV and *vice versa*.

2.1.3. Validation as a vaccine strain

Tests for immunogenicity of the EHV-1/4 MSV stocks should be performed in horses on an experimental test vaccine prepared from the highest passage level of the MSV allowed for use in vaccine production. The test for MSV immunogenicity consists of vaccination of horses with low antibody titres to EHV-1/4, with doses of the test vaccine that will be recommended on the final product label. Second serum samples should be obtained and tested for significant increases in neutralising antibody titre against the virus, 21 days after the final dose.

Samples of each lot of MSV to be used for preparation of live attenuated ER vaccines must be tested for safety in horses determined to be susceptible to the virulent wild-type virus, including pregnant mares in the last 4 months of gestation. Vaccine safety must be demonstrated in a ‘safety field trial’ in horses of various ages from three different geographical areas. The safety trial should be conducted by independent veterinarians using a prelicensing batch of vaccine. EHV-1 vaccines making a claim for efficacy in controlling abortion must be tested for safety in a significant number of late gestation pregnant mares, using the vaccination schedule that will be recommended by the manufacturer for the final vaccine product.

2.2. Method of manufacture

2.2.1. Procedure

A detailed protocol of the methods of manufacture to be followed in the preparation of vaccines for ER must be compiled, approved, and filed as an Outline of Production with the appropriate licensing agency. Specifics of the methods of manufacture for ER vaccines will differ with the type (live or inactivated) and composition (EHV-1 only, EHV-1 and EHV-4, EHV-4 and equine influenza viruses, etc.) of each individual product, and also with the manufacturer.

2.2.2. Requirements for ingredients

Cells, virus, culture medium, and medium supplements of animal origin that are used for the preparation of production lots of vaccine must be derived from bulk stocks that have passed the prescribed tests for bacterial, fungal, and mycoplasma sterility; nontumorgenicity; and absence of extraneous viral agents.

2.2.3. Final product batch tests

i) Sterility

Samples taken from each batch of completed vaccine are tested for bacteria, fungi, and mycoplasma contamination. Procedures to establish that the vaccine is free from extraneous viruses are also required; such tests should include inoculation of cell cultures that allow detection of the common equine viruses, as well as techniques for the detection of BVDV and PPV in ingredients of animal origin used in the production of the batch of vaccine.

ii) Identity

Identity tests shall demonstrate that no other vaccine strain is present when several strains are propagated in a laboratory used in the production of multivalent vaccines.

iii) Safety

Safety tests shall consist of detecting any abnormal local or systemic adverse reactions to the vaccine in the host species by all vaccination route(s). Tests to assure safety of each production batch of ER vaccine must demonstrate complete inactivation of virus (for inactivated vaccines) as well as a level of residual virus-killing agent that does not exceed the maximal allowable limit (e.g. 0.2% for formaldehyde).

iv) Batch potency

Batch potency is examined on the final formulated product. Batch control of antigenic potency for EHV-1 vaccines only may be tested by measuring the ability of dilutions of the vaccine to protect hamsters from challenge with a lethal dose of hamster-adapted EHV-1 virus. Although potency testing on production batches of ER vaccine may also be performed by vaccination of susceptible horses followed by assay for seroconversion, the recent availability of virus type-specific MAbs has permitted development of less costly and more rapid *in-vitro* immunoassays for antigenic potency. The basis for such *in-vitro* assays for ER vaccine potency is the determination, by use of the specific MAb, of the presence of at least the minimal amount of viral antigen within each batch of vaccine that correlates with the required level of protection (or seroconversion rate) in a standard animal test for potency.

2.3. Requirements for authorisation/registration/licencing

2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C.2.1 and C.2.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

2.3.2 Safety requirements

Vaccine safety should be evaluated in vaccinated animals using different assays (see Section 2.2.3.iii).

2.3.3 Efficacy requirements

Vaccine efficacy (protection) is estimated in vaccinated animals directly by evaluating their resistance to live pathogen challenge.

2.3.4 Duration of immunity

As part of the licensing or marketing authorisation procedure, the manufacturer may be required to demonstrate the duration of immunity (DOI) of a given vaccine by either challenge or alternative test at the end of the claimed period of protection.

Tests to establish the duration of immunity to EHV-1/4 achieved by immunisation with each batch of vaccine are not required. The results of many reported observations indicate that vaccination-induced immunity to EHV-1/4 is not more than a few months in duration; these observations are reflected in the frequency of revaccination recommended on ER vaccine product labels.

2.3.5 Stability

As part of the licensing or marketing authorisation procedure, the manufacturer will be required to demonstrate the stability of all the vaccine's properties at the end of the claimed shelf-life period. Storage temperature shall be indicated and warnings should be given if product is damaged by freezing or ambient temperature.

At least three production batches of vaccine should be tested for shelf life before reaching a conclusion on the vaccine's stability. When stored at 4°C, inactivated vaccine products generally maintain their original antigenic potency for at least 1 year. Lyophilised preparations of the live virus vaccine are also stable during storage for 1 year at 4°C. Following reconstitution, live virus vaccine is unstable and cannot be stored without loss of potency.

Note: current vaccines are authorised for prevention of respiratory disease or as an aid in the prevention of abortion. Unless the vaccine's ability to prevent neurological disease is under investigation, the virus used in the challenge experiments should not be a strain with a history of inducing neurological disease.

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

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2017.