CHAPTER 2.5.9.

EQUINE RHINOPNEUMONITIS
(EQUINE HERPESVIRUS 1 AND 4)

SUMMARY

Equine rhinopneumonitis (ER) is a collective term for any one of several highly 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 very many domestic equine populations worldwide. Primary infection by either EHV-1 or EHV-4 is characterised by a primary 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 rare 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 reactivate following stress or pregnancy. Most horses will be infected during their lifetime. Detection of viral DNA or anti-EHV antibodies should therefore be interpreted with care. Infections by EHV-1 in particular are capable of progressing beyond the respiratory mucosa to cause the more serious disease manifestations of abortion, perinatal foal death, or neurological dysfunction.

Identification of the agent: The standard method of identification of EHV-1 and EHV-4 as the herpesviral agents of ER continues to be laboratory isolation of the virus from appropriate clinical or necropsy material by polymerase chain reaction (PCR), followed by seroconfirmation of its identity. 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 samples 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 disease infection. Unlike EHV-4, EHV-1 will also grow on in other cell types such as RK-13s and this property can be used to distinguish between the two viruses. Positive identification of viral isolates as EHV-1 or EHV-4 can be achieved by immunofluorescence with type-specific monoclonal antibodies.

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

Sensitive and reliable methods for EHV-1/4 detection by polymerase chain reaction or immunoperoxidase staining have been developed and are useful adjuncts to standard virus cultivation techniques for diagnosis of ER.

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

Serological tests: Because most horses will 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 sufficient for a positive diagnosis of recent infection active ER. 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 enzyme-linked immunosorbent assay, or complement fixation (CF). Neither of these assays is type-specific
but both have proven useful for diagnostic purposes as VN and CF, and to a lesser extent VN, antibodies are 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 and diagnostic biologicals: Both live attenuated and inactivated viral vaccines of varying composition are commercially available for use in assisting in the control of EHV-1/4-ER. While vaccination is helpful in reducing the incidence of abortion in mares, and in ameliorating severity of clinical signs 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 strict adherence to the well-established tenets of sound management practices known to reduce the risk of infection.

Revaccination at frequent intervals is recommended with 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 ER-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 use. 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.

ER is highly contagious among susceptible horses, with viral transmission to cohort animals occurring by inhalation of aerosols of virus-laden respiratory secretions. Mortality 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 represents a major source of infection. Extensive use of vaccines has not eliminated EHV infections, and the world-wide annual financial burden 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. 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 pattern, the respiratory infection is a frequent and significant cause of interrupted schedules among horses assembled for training, racing, or competitive 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 and/or central nervous system (CNS) disease are not eliminated. Like other herpesviruses, EHV-1/4 cause long-lasting latent infections and latently infected horses represent an infection risk for other horses. Virus can reactivate 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 marker 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 useful to be aware of an increased risk of neurological complications. However,
for practical purposes strain-typing does not influence the requirement for strict management practices during an outbreak of EHV-1—is not relevant for agent identification, or international trade. However, it can be with reference to biosecurity practices implemented in management during outbreak management of outbreaks of equine herpesvirus myeloencephalopathy.

**B. DIAGNOSTIC TECHNIQUES**

Both EHV-1 and EHV-4 because ER is a have the potential to be highly contagious disease with viruses and the former occasionally can occurring as causes explosive outbreaks with high mortality from abortion and neonatal or neurological sequelae-disease. Rapid diagnostic methods are therefore useful for managing the disease. Polymerase chain reaction (PCR) and quantitative PCR (qPCR) 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. Although several and innovative diagnostic techniques based on enzyme linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), immunohistochemical staining with peroxidase, or nucleic acid hybridisation probes have been recently described, their use is often restricted to specialised reference laboratories, and thus the method of choice for diagnosis of ER by diagnostic virology laboratories handling many routine samples continues to be the traditional methodology of cell culture isolation followed by sero-identification of the isolated viruses. Successful laboratory isolation of EHV-1/4 depends on strict adherence to proper methods for both sample collection and laboratory processing.

**Table 1. Test methods available and their purpose**

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<thead>
<tr>
<th>Method</th>
<th>Purpose</th>
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<tr>
<td></td>
<td>Population freedom from infection</td>
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<tr>
<td>Agent identification¹</td>
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<td>Virus isolation</td>
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<td>PCR</td>
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<td>Detection of immune response²</td>
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<td>VN</td>
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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 standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

PCR = polymerase chain reaction; AGID = agar gel immunodiffusion; CFT = complement fixation test; VN = virus neutralisation; ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent (Allen et al., 2004)

1 A combination of agent identification methods applied on the same clinical sample is recommended.
2 One of the listed serological tests is sufficient.
1.1. Collection and preparation of samples

Samples of nasopharyngeal exudate for virus isolation. 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 swabbing the nasopharyngeal area with a 5 × 5 cm gauze sponge attached to the end of a 50 cm length of flexible, stainless steel wire encased in latex rubber tubing. A guarded uterine swab device can also be used sampling the area with a swab of an appropriate size and length for horses. After collection, the swab should be removed from the wire and transported immediately to the virology laboratory in 3 ml of cold (not frozen) fluid 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).

Virological examination of fetal. Tissue samples: total DNA can be extracted using a number of commercially available kits and used in PCR to detect viral DNA (described below Section B.1.2.i). Virus isolation from placenta and fetal tissues from suspect cases of EHV-1 abortion is most successful when performed on aseptically collected samples of placenta, liver, lung, thymus and spleen. The 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. In ante-mortem cases of EHV-1 neurological disease, the virus can often be isolated from the leukocyte fraction of the blood of acutely infected horses or, less often, from the nasopharynx of the affected animal or cohort animals. For attempts at virus isolation from blood leukocytes, a 20 ml sample of sterile blood, collected in citrate, or heparin anticoagulant (EDTA [ethylene diamine tetra-acetic acid] should not be used as it can destroy cell cultures). The samples should be transported without delay to the laboratory on ice, but not frozen. Although the 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 PCR examination—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 attempts at virus isolation from blood leukocytes, take collect a 20 ml sample of sterile blood, collected using aseptic technique in citrate, or heparin anticoagulant or EDTA (ethylene diamine tetra-acetic acid) anticoagulant should not be used as it can destroy cell cultures however 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 -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 epizootic outbreaks of abortion or respiratory tract neurological disease in which a rapid identification of the virus is critical for guiding management strategies including movement restrictions. PCR examinations of spinal cord and brain tissue, as well as peripheral blood leukocytes (PBMC), are important in seeking a diagnosis on a horse with neurological signs. However, the interpretation of the amplification by PCR of genomic fragments of EHV-1 or EHV-4 in lymph nodes or trigeminal ganglia from adult horses is complicated by the high prevalence of latent EHV-1 and EHV-4 DNA in such tissues (Welch et al., 1992).

PCR technology is evolving rapidly and a variety of assays has 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 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 neuropathic 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.

A simple 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.) is described here (Borchers & Slater, 1993).

Real-time PCR, based on the TaqMan® method, has become the method of choice for many diagnostic tests and provides rapid and sensitive detection of viral DNA. RT-PCR assays have been described for EHV-1 & 4. The qPCR 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 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 G (gG) for EHV-1 and glycoprotein B (gB) for EHV-4. Discrimination between EHV-1 and EHV-4 is carried out by the incorporation of type-specific dual labelled TaqMan™ probes. The method utilizes in-house designed primers and probes, based on methods published by Hussey et al (2006) and Lawrence et al (1994). To establish such a qPCR 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.

i) Suitable specimens

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

ii) Primers and probes

| EHV 1 Forward: | GGGTTCTTAATTGCATTCAGACC |
| EHV 1 Reverse: | GTAGGTGCGGTTAGATCTCACAAG |
| EHV 4 Forward: | TAGCAAACACCCACTAATAATAGCAAG |
| EHV 4 Reverse: | GCTCAAATCTCTTTATTTTATGTCATATGC |
| EHV1gB/probe: | {FAM}TCTCCAACGAACTCGCCAGGCTGTACC{BHQ1} |
| EHV4gC/probe: | {JOE}CGGAACAGGAACTCACTTCAGAGCCAGC{BHQ1} |

iii) RT-PCR standards

A DNA standard curve should be used to quantify the levels of viral DNA, comprising at least 4 standards containing EHV-1 & 4 target DNA at known concentrations. All standards should be diluted in 1ng/ml Polynosinic–polycytidylic acid (Polyl/C) to stabilise the DNA in solution. These should be stored at -20C 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 RT-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 a 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.

### Test procedure

1. **Prepare template DNA from test specimens:** following sample homogenisation and lysis in the presence of a chaotropic salt, nucleic acids bind selectively to silica or cationic resin substrates. Substrate-bound nucleic acids are purified in a series of rapid wash steps followed by recovery with low-salt elution. The reagents for performing such steps for rapid nucleic acid isolation are available in kit format from a number of commercial sources.

#### Nested primer sequences specific for EHV-1

1. **BS-1-P1 = 5’-TCT-ACC-CCT-ACC-ACT-CCT-TC-3’ (917–936)**
2. **gB1-R2 = 5’-ACC-CTG-TCG-ATG-TGG-TAA-AAC-CTG-AGA-G-3’ (2390–2363)**
3. **BS-1-P3 = 5’-CTT-TAG-CGG-TGA-TGT-GGA-AT-3’ (1377–1396)**
4. **gB1-R3 = 5’-AAG-TAG-CGC-TTC-TGA-TTG-AGG-3’ (2414–2423)**

#### Nested primer sequences specific for EHV-4

1. **BS-4-P1 = 5’-TCT-ATT-GAG-TTG-GCT-ATG-CT-3’ (1705–1724)**
2. **BS-4-P2 = 5’-TCC-TGG-TTG-TTA-TTG-GGT-AT-3’ (2656–2637)**
3. **BS-4-P3 = 5’-TCT-TTC-GCC-CAC-TCT-TGA-CG-3’ (1857–1876)**
4. **BS-4-P4 = 5’-ACT-GCC-TCT-CCC-ACC-TTA-CC-3’ (2456–2437)**

#### PCR conditions for first stage amplification: specimen template DNA (1–2 µg in 2 µl) is added to a PCR mixture (total volume of 50 µl) containing 1 x PCR buffer (50 mM KCl, 40 mM Tris/HCl pH 9.0, 0.1% Triton X-100), 200 µM of each deoxynucleotide triphosphate (dNTP), 2.5 mM MgCl$_2$, 2.0 µM of each outer primer (BS-1-P1 and gB1-R2) for EHV-1 detection and, in a separate reaction mixture, BS-4-P1 and BS-4-P2 for EHV-4 detection, and 0.5 u Taq DNA polymerase. Cycling parameters are: initial denaturation at 94°C for 4 minutes; 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 90 seconds; with a final extension at 72°C for 10 minutes. Separate reaction mixtures containing either known viral DNA or no DNA (water) should be prepared and amplified as positive and negative controls.

#### PCR conditions for second stage (nested) amplification: two µl of a 1/10 dilution of the first amplification product is added to a fresh PCR mixture (total-volume of 50 µl) containing 1 x PCR buffer, 200 µM of each dNTP, 2.5 mM MgCl$_2$, 2.0 µM of each nested primer (BS-1-P3 and gB1-R-a for EHV-1 detection and, in a separate reaction mixture, BS-4-P3 and BS-4-P4 for EHV-4 detection) and 0.5 u Taq DNA polymerase. Cycling parameters are: initial denaturation at 94°C for 4 minutes; 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes.

#### Gel analysis of amplified products: 10 µl of each final amplified product, including controls, is mixed with 2 µl of 6 x loading dye and electrophoresed on a 1.5% agarose gel in Tris-borate or Tris-Borate running buffer, along with a 100 base pairs (bp) DNA ladder. Amplified products are detected using a suitable DNA stain, of either 770 bp for EHV-1 or 580 bp for EHV-4.

### 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 cell strains of 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 then filtered through a sterile, 0.45 µm membrane syringe filter unit into a second sterile tube. Filtration will decrease if heavy bacterial contamination is expected, but this may also lower virus titre. Recently prepared cell monolayers in 25 cm$^2$ tissue culture flasks are inoculated with 0.5 ml of 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 on a platform rocker for 1.5–2 hours. Monolayers of uninoculated control cells should be incubated in parallel with sterile transport medium only.

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 5 ml of supplemented maintenance medium (MEM containing 2% fetal calf serum [FCS] and twice the standard concentrations of antibiotics [penicillin, streptomycin, gentamicin, and amphotericin B]), the flask 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 from the tissues of aborted fetuses or from post-mortem cases of neurological disease (e.g. rabbit kidney [RK-13 [AAAT–CC13]], 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 which can cause abortion, but equine-derived cell cultures are most sensitive and must be used if the CPE is absent or not characteristic. Infections in the neurological marker can be completed at some laboratories. Isolates from positive cultures should be submitted to an OIE reference laboratory to maintain a virus isolate bank. 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 completed at some laboratories.

**Blood samples**: Both EHV-1 and, less commonly, EHV-4 can be isolated from PBMC. Culture of peripheral blood leukocytes PBMC for the presence of EHV-1 can be attempted from horses during the early stages of myeloencephalopathy, neurological disease. Buffy coats may be prepared from unclotted blood by centrifugation at 600 g for 15 minutes, and theuffy coat is taken after the plasma has been carefully removed. Theuffy coat is then layered onto Ficoll 1.077 g/ml (commercially available) and centrifuged at 400 g for 20 minutes and the leukocyte-rich interface is then layered onto Ficoll 1.077 and centrifuged in the same way. The PBMC interface (without the granulocytes) is washed twice in (300 g for 10 minutes) and resuspended in 1 ml of MEM containing 2% FCS. Then, 0.5 ml is inoculated into duplicate cell monolayers in 25 cm² 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.

**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 completed at some laboratories.**
1.4. Virus detection by direct immunofluorescence

Direct immunofluorescent detection of EHV-4 antigens in samples of post-mortem tissues collected from aborted equine fetuses and the placenta provides an indispensable method to the veterinary diagnostic laboratory for making a rapid preliminary diagnosis of herpesvirus abortion (Gunn, 1992). Side-by-side comparisons of the immunofluorescent and cell culture isolation techniques on more than 100 cases of equine abortion have provided evidence that the diagnostic reliability of direct immunofluorescent staining of fetal tissues obtained at necropsy this technique approaches that of virus isolation attempts from the same tissues.

In the United States of America (USA), specific and potent polyclonal antiserum to EHV-1, prepared in swine and conjugated with FITC, is provided 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 media 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

Enzyme-immunohistochemical (IH) staining methods, such as immunoperoxidase, have been developed recently as procedures for detecting EHV-1 antigen in paraffin-embedded fixed tissues of aborted equine fetuses, placental tissues or neurologically affected horses (Schultheiss et al., 1993; Whitwell et al., 1992). Such ancillary IH techniques for antigen detection may facilitate identification of the virus in can be used as an alternative to immunofluorescence described above and can also be readily applied to archival tissue samples or in clinical cases in which traditional laboratory methods for EHV-1 detection have been unsuccessful. Immunoenzymatic-immunohistochemical staining for EHV-1 is particularly useful for the simultaneous evaluation of morphological lesions and the identification of the virus infectious agent. 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 4, however it provides a useful method for rapid diagnosis of EHV-induced abortion.
et al., 2001). Diagnosis of ER by PCR is rapid, sensitive, and does not depend on the presence of infectious virus in the clinical sample. It now forms an integral part of a range of diagnostic tests currently available for ER, each with its own advantages and limitations.

For diagnosis of active infection by EHV, PCR methods are most reliable with samples from aborted fetuses or from nasopharyngeal swabs and peripheral blood leukocytes of foals and yearlings; they are most useful in explosive epizootics of abortion or respiratory tract disease in which a rapid identification of the virus is critical for guiding management strategies. PCR examinations of spinal cord and brain tissue, as well as PBMC, are important in seeking a diagnosis on a horse with neurological signs. However, the interpretation of the amplification by PCR of genomic fragments of EHV-1 or EHV-4 in lymph nodes or trigeminal ganglia from adult horses is complicated by the high prevalence of latent EHV-1 and EHV-4 DNA in such tissues (Welch et al., 1992).

A simple multiplex PCR assay for simultaneous detection of both EHV-1 and EHV-4 has been described (Wagner et al., 1993). A more sensitive protocol for nested PCR detection of EHV-1 or EHV-4 in clinical or pathological specimens (nasal secretions, blood leukocytes, brain and spinal cord, fetal tissues, etc.) is described here (Borchers & Slater, 1993). This procedure has been used successfully; however, the technology in this area is changing rapidly and other simpler more sensitive techniques are becoming available.

i) Prepare template DNA from test specimens: Following sample homogenisation and cell (and virion) lysis in the presence of a chaotropic salt, nucleic acids bind selectively to silica or cationic resin substrates. Substrate bound nucleic acids are purified in a series of rapid wash steps followed by recovery with low salt elution. The reagents for performing such steps for rapid nucleic acid isolation are available in kit format from a number of commercial sources (e.g., High Pure PCR Template Preparation Kit, Roche Molecular Biochemicals, Indianapolis, USA; QIAamp DNA Kit, Qiagen, Valencia, USA).

ii) Nested primer sequences specific for EHV-1 (based on those described in Borchers & Slater, 1993):

BS-1 P1 = 5'-TCT-ACC-CCT-ACG-ACG-CTT-TC-3' (917–936)
gB1R-2 = 5'-ACC-CTG-TGC-ATC-TCC-TAA-AAC-CTG-AGA-G-3' (2390–2363)
BS-1 P3 = 5'-CTT-CTA-GGG-TTA-GGTT-GGA-AT-3' (1377–1396)
gB1R-a = 5'-AAG-TAG-CCC-CTC-TGA-TTG-AGG-3' (2147–2127)

iii) Nested primer sequences specific for EHV-4 (Borchers & Slater, 1993):

BS-4 P1 = 5'-TCT-ATT-GAG-TCT-GTG-ATG-CT-3' (1705–1724)
BS-4 P2 = 5'-TCC-TGG-TTG-TTA-TTG-GGT-AT-3' (2656–2637)
BS-4 P3 = 5'-TGT-CTC-CCC-CAC-TCT-TGA-CG-3' (1857–1876)
BS-4 P4 = 5'-ACT-GCC-TCT-CCC-ACC-TTA-CG-3' (2456–2437)

iv) PCR conditions for first stage amplification: Specimen template DNA (1–2 µg in 2 µl) is added to a PCR mixture (total volume of 50 µl) containing 1× PCR buffer (50 mM KCl, 10 mM Tris/HCl, pH 9.0, 0.1% Triton X-100), 200 µM of each deoxynucleotide triphosphate (dNTP), 2.5 mM MgCl2, 2.0 µM of each outer primer (BS-1 P1 and gB1R-2) for EHV-1 detection and, in a separate reaction mixture, BS-1 P1 and BS-4 P2 for EHV-4 detection) and 0.5 µ l Tag DNA polymerase. Cycling parameters are: initial denaturation at 94°C for 4 minutes; 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 90 seconds, with a final extension at 72°C for 10 minutes. Separate reaction mixtures containing either known viral DNA or no DNA (water) should be prepared and amplified as positive and negative controls.

v) PCR conditions for second stage (nested) amplification: Two µ l of a 1/10 dilution of the first amplification product is added to a fresh PCR mixture (total volume of 50 µl) containing 1× PCR buffer, 200 µ M of each dNTP, 2.5 mM MgCl2, 2.0 µ M of each nested primer (BS-1 P3 and BS-4 P3 for EHV-1 detection and, in a separate reaction mixture, BS-1 P3 and BS-4 P4 for EHV-4 detection) and 0.5 µ l Tag DNA polymerase. Cycling parameters are: initial denaturation at 94°C for 4 minutes; 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute; with a final extension at 72°C for 10 minutes.
vi) Gel analysis of amplified products: 10 µl of each final amplified product, including controls, is mixed with 2 µl of 6× loading dye and electrophoresed on a 1.5% agarose gel in Tris-acetate or Tris-Borate running buffer, along with a 100 base pairs (bp) DNA ladder. The gel is stained with ethidium bromide and viewed by UV transillumination for amplified products of either 770 bp for EHV-1 or 580 bp for EHV-4.

1.6. Histopathology

Histopathological examination of sections of formalin-fixed paraffin-embedded tissues—placenta and lung, liver, spleen, adrenal and thymus from aborted fetuses and brain and spinal cord from neurologically affected horses should be carried out as an essential part of the laboratory diagnosis of these two clinical manifestations of ER. In aborted fetuses, typical herpetic 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 pathognomonic lesions for EHV-1. The characteristic but not pathognomonic, 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

Because of the ubiquity of the viral agents of ER and the high seroprevalence among horses in most parts of the world, the demonstration of a negative antibody titre to EHV-1/4 by serological testing of horses designated for export is not part of present veterinary regulations that seek to prevent international spread of infectious diseases of horses. Serological testing can, however, be a useful adjunct procedure for assisting in the diagnosis of ER in horses. Serodiagnosis of ER EHV-1 and 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 for rising antibody titres against EHV-1/4 may provide information 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—rare cases of virologically negative fetuses aborted as a result of EHV-1 infection; in some cases, The EHV 1/4 nucleic acid genome may be identified from these tissues by PCR.

Serum antibody levels to EHV-1/4 may be determined by ELISA (Dutta et al., 1983). Virus neutralisation (VN) (Thomson et al., 1976), complement fixation (CF) tests (Thomson et al., 1976) or ELISA (Dutta-Crabbe 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; antibody titre determinations on the same serum may differ from one laboratory to another. Furthermore, the CF and VN all of the serological tests mentioned detect antibodies that are cross-reactive between EHV-1 and EHV-4. Nonetheless, the demonstration by any of the tests, 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. Type specific ELISA kits are available commercially. The ELISA and CF test have the advantage that they provide results faster and do not require cell culture facilities. Recently, a type-specific ELISA that can distinguish between antibodies to EHV-1 and EHV-4 was developed and made commercially available (Crabbe et al., 1995). 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 serological 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
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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 cells/ml. Note that RK-13 cells can be used with EHV-1 but do not give clear 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. This 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 to 1/256.

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% CO2 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% CO2 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 AND DIAGNOSTIC BIOLOGICALS

NB: SECTION C IS “UNDER STUDY”. THIS IS THE LAST ADOPTED VERSION PUBLISHED IN 2008

Both live attenuated and inactivated vaccines are available as licensed, commercially prepared products for use as prophylactic aids in reducing the burden of disease in horses caused by EHV-1/4 infection. Clinical experience has demonstrated that none of the vaccine preparations should be relied on to provide an absolute degree of
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Protection from ER. Multiple doses repeated annually, of each of the currently marketed ER vaccines are recommended by their respective manufacturers. Vaccination schedules vary with the particular vaccine.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.6 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

At least sixteen vaccine products for ER, each containing different permutations of EHV-1, EHV-4, and the two subtypes of equine influenza virus, are currently marketed by five veterinary biologicals manufacturers.

The clinical indications stated on the product label for use of the several available vaccines for ER are either herpesvirus-associated respiratory disease, 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 conclusively demonstrated to prevent the occurrence of neurological disease sometimes associated with EHV-1 infection.

1. Seed management

1.1. Characteristics and culture

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, 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. 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.

1.2. Validation as a vaccine

1.2.1. Purity

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 rhinovirus, 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.

1.2.2. Safety

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.

1.2.3. Immunogenicity

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.

1.2.4. Efficacy

An important part of the validation process is the capacity of a prelicensing lot of the ER vaccine to provide a significant level of clinical protection in horses from the particular disease
manifestation of EHV-1/4 infection for which the vaccine is offered, when used under the
conditions recommended by the manufacturer's product label. Serological data are not
acceptable for establishing the efficacy of vaccines for ER. Efficacy studies must be designed to
ensure appropriate randomisation of test animals to treatment groups, blinding of the recording
of clinical observations, and the use of sufficient numbers of animals to permit statistical
evaluation for effectiveness in prevention or reduction of the specified clinical disease. The
studies should be performed on fully formulated experimental vaccine products (a) produced in
accordance with, (b) at or below the minimum antigenic potency specified in, and (c) produced
with the highest passage of MSV and MCS allowed by the approved ‘Outline of Production’ (see
Section C.2). Vaccine efficacy is demonstrated by vaccinating a minimum of 20 EHV-1/4-
susceptible horses possessing serum neutralising antibody titres ≤32, followed by challenge of
the vaccinates and ten nonvaccinated control horses with virulent virus. A significant difference
in the clinical signs of ER must be demonstrated between vaccinates and nonvaccinated control
horses. The vaccination and challenge study must be performed on an identical number of
pregnant mares and scored for abortion if the vaccine product will make a label usage claim ‘for
prevention of’ or ‘as an aid in the prevention of’ abortion caused by EHV-1.

2. Method of manufacture

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.

3. In-process control

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.

4. Batch control

Each bulk production lot of ER vaccine must pass tests for sterility, safety, and immunogenic potency.

4.1. 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.

4.2. Safety

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). Safety testing in laboratory animals is also
required.

4.3. Potency

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 either viral challenge or assay for seroconversion the
recent availability of virus type-specific MAbs has permitted development of less costly and more rapid
\textit{in-vitro} immunoassays for antigenic potency. The basis for such \textit{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.

4.4. Duration of immunity
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.

4.5. Stability

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.

5. Tests on the final product

Before release for labelling, packaging, and commercial distribution, randomly selected filled vials of the final vaccine product must be tested by prescribed methods for freedom from contamination and safety in laboratory test animals.

5.1. Safety

See Section C.4.2.

5.2. Potency

See Section C.4.3.

REFERENCES


Chapter 2.5.9. – Equine rhinopneumonitis


NB: There are OIE Reference Laboratories for Equine rhinopneumonitis (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/).

Please contact the OIE Reference Laboratories for any further information on...
diagnostic tests, reagents and vaccines for equine rhinopneumonitis and to submit strains for further characterisation.