CHAPTER 3.6.8.

EQUINE RHINOPNEUMONITIS (INFECTION WITH VARICELLOVIRUS EQUIDALPHA1)

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, formally known as equid herpesvirus-1 and -4 (EHV-1 and EHV-4). Infection with EHV-1 is listed by WOAH and is therefore the focus of this chapter. The classification of the virus has been reviewed and EHV-1 is now known as Varicellovirus equidalpha1. For the purposes of the chapter, the acronym EHV-1 will continue to be used. EHV-1 is endemic in most domestic equine populations worldwide.

Primary infection by EHV-1 is characterised by upper respiratory tract disease of varying severity that is related to the age and immunological status of the infected animal. Following viraemia EHV-1 may also cause the more serious complications of abortion, perinatal foal death, or paralytic neurological disease (equine herpesvirus myeloencephalopathy). Like other herpesviruses, EHV-1 induces long-lasting latent infections and can be reactivated following stress. Furthermore, most horses are likely to be re-infected multiple times during their lifetime, often subclinically.

Identification of the agent: The standard method of identification of EHV-1 from appropriate clinical or necropsy material is by polymerase chain reaction (PCR).

Positive identification of viral isolates as EHV-1 can be achieved by type-specific PCR or sequencing. Viruses can be isolated in cell culture from the following sample types: nasal or nasopharyngeal swab extracts taken from horses with acute respiratory tract infection, the placenta, liver, lung, spleen, adrenal glands or thymus of aborted fetuses and early foal deaths, and from the leukocyte fraction of the blood of animals during the febrile stage. A rapid presumptive diagnosis of abortion induced by EHV-1 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 other diagnostic techniques.

Serological tests: As 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 not confirmation 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.

Requirements for vaccines: Both live attenuated and inactivated viral vaccines are available for use in assisting in the control of EHV-1. 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 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 *et al*., 1999). The disease is recognised as a threat to the international horse industry, and is caused by either of two members of the *Herpesviridae* family, formerly known as equid alphaherpesvirus-1 and -4 (EHV-1 and EHV-4). The viruses are now classified as Varicellovirus equidalpha1 and Varicellovirus equidalpha4. For the purposes of the chapter, the acronyms EHV-1 and EHV-4 will continue to be used. 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*., 1998). With the exception of EHV-1 in Iceland (Thorsteinsdóttir *et al*., 2021), the two herpesviruses are considered endemic 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 WOAH and is therefore the focus of this chapter.

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-1 infections, and the world-wide financial impact from this equine pathogen is considerable.

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 virus infects and multiplies 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. Bi-phasic fever, viraemia and complications are more likely with EHV-1 than EHV-4. Subclinical infections with EHV-1 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 after several months. Although reinfections cause less severe or clinically inapparent respiratory disease, the risks of subsequent abortion or neurological disease remain. Like other herpesviruses, EHV-1 causes 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. 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. ER abortions occur annually in horse populations worldwide and may be sporadic or multiple. Foals infected *in utero* may be born alive and die within a few days of birth. EHV-1 neurological disease is less common than abortions but has been recorded all over the world with associated fatalities. Outbreaks result in movement restrictions and, sometimes, cancellation of equestrian events (Couroucé *et al*., 2023; FEI, 2021).

Strain typing has been shown to be not reliable for predicting the clinical outcome of EHV-1 infection but can be useful in epidemiological investigations (Garvey *et al*., 2019; Nugent *et al*., 2006; Sutton *et al*., 2019).

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B. DIAGNOSTIC TECHNIQUES

EHV-1 is transmitted by the respiratory route and has the potential to be highly contagious particularly where large numbers of horses are housed in the same air space. EHV1 can cause explosive outbreaks of abortion or

neurological disease. Rapid diagnostic methods are therefore essential for managing the disease. Real-time polymerase chain reaction (PCR) assays are routinely used by diagnostic laboratories worldwide and are both rapid and sensitive. Real-time PCR assays have been developed for both detection of EHV-1 and quantification of viral load and have replaced virus isolation as the frontline diagnostic test in the majority of laboratories. Virus isolation can still be useful, in cases 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 are employed in some laboratories for rapid diagnosis of EHV-induced abortion from fresh or embedded tissue. Virus neutralisation (VN) and complement fixation test (CFT) are the most frequently used serological tests, and seroconversion in paired samples is considered indicative of exposure to virus by natural infection or by vaccination.

Table 1. Test methods available for the diagnosis of infection with EHV-1 and their purpose

Method	Purpose					
	Population freedom from infection[a]	Individual animal freedom from infection prior to movement[b]	Contribute to eradication policies[c]	Confirmation of clinical cases[d]	Prevalence of infection - surveillance[e]	Immune status in individual animals or populations post-vaccination[f]
Identification of the agent[g]						
Virus isolation	–	++	–	++	–	–
PCR	–	+++	–	+++	–	–
Direct immunofluorescence	–	–	–	++	–	–
Detection of immune response						
VN	++	++	–	++	+++	+++
ELISA	+	++	–	++	++	++
CFT	–	++	–	++	–	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; VN = virus neutralisation;
ELISA = enzyme-linked immunosorbent assay; CFT = complement fixation test.
[a]See Appendix 1 of this chapter for justification table for the scores given to the tests for this purpose.
[b]See Appendix 2 of this chapter for justification table for the scores given to the tests for this purpose.
[c]No eradication policies exist for equine rhinopneumonitis.
[d]See Appendix 4 of this chapter for justification table for the scores given to the tests for this purpose.
[e]See Appendix 5 of this chapter for justification table for the scores given to the tests for this purpose.
[f]See Appendix 6 of this chapter for justification table for the scores given to the tests for this purpose.
[g]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection 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 acute stage 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. phosphate buffered saline [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). 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, adrenal glands 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 are often unsuccessful; however, these samples 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 detection by PCR or isolation from blood leukocytes, collect a 10–20 ml sample of blood, using an aseptic technique in, heparin or EDTA [ethylene diamine tetra-acetic acid] anticoagulant. EDTA is the preferred anticoagulant for PCR testing in some laboratories as heparin may inhibit DNA polymerase. The samples should be transported without delay to the laboratory on ice, but not frozen.

Cerebrospinal fluid: the detection of EHV-1 DNA in cerebrospinal fluid has been reported in cases of neurological disease.

1.2. Virus detection by polymerase chain reaction

Diagnosis by PCR is rapid, sensitive, and does not depend on the presence of infectious virus in the clinical sample. PCR methods are routinely used to detect EHV-1 DNA in nasopharyngeal swabs and tissue samples. They are particularly useful in explosive outbreaks of abortion, respiratory or neurological disease, in which rapid identification and monitoring of the virus spread 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 (Pronost *et al.*, 2012).

Real-time (or quantitative) PCR has become the method of choice for the majority of diagnostic laboratories and provides rapid and sensitive detection of viral DNA. 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), buffy coat, tracheal wash (TW) or broncho-alveolar lavage (BAL) are all suitable. DNA should be extracted using an appropriate kit or robotic system.

There is no internationally standardised real-time PCR method for EHV-1 but Table 2 summarises the primer and probe sequences for some of the most widely used assays. Type-specific PCR primers have been designed to distinguish between the presence of EHV-1 and EHV-4. The optimised thermocycler times and temperatures are documented in the publications cited.

Table 2. Primer and probe sequences for EHV1 detection by real-time PCR

Primer	Primer sequence (5′ to 3′)	Target	Reference
Forward	CAT-ACG-TCC-CTG-TCC-GAC-AGA-T	EHV-1 gB	Hussey *et al.,* 2006
Reverse	GGTACTCGGCCTTTGACGAA		
Probe	FAM-TGA-GAC-CGA-AGA-TCT-CCT-CCA-CCG-A-BHQ1		
Forward	TAT-ACT-CGC-TGA-GGA-TGG-AGA-CTT-T	EHV-1 gB	Pusterla *et al.,* 2009
Reverse	TTG-GGG-CAA-GTT-CTA-GGT-GGT-T		
Probe	6FAM-ACA-CCT-GCC-CAC-CGC-CTA-CCG		
Forward	GCG-GGC-TCT-GAC-AAC-ACA-A	EHV-1 gC	
Reverse	TTG-TGG-TTT-CAT-GGG-AGT-GTG-TA		

Primer	Primer sequence (5′ to 3′)	Target	Reference
Probe	FAM-TAA-CGC-AAA-CGG-TAC-AGA-A-BHQ1		ISO 17025 accredited for the detection of EHV-1 at WOAH Reference Laboratory

*Reference material and sample panels for real-time PCR can be obtained from the WOAH Reference Laboratories.

- **Point of care (POC) molecular tests**

Loop-mediated isothermal amplification (LAMP) assays for the detection of EHV-1 have been described (Nemoto *et al*., 2011). An evaluation of a hydrolysis probe-based insulated isothermal PCR (iiPCR) assay for the detection of EHV-1 showed it to have a high sensitivity and specificity compared with real-time PCR (Balasuriya *et al*., 2017). However further validation of POC tests in the field is required.

- **Molecular characterisation**

Allelic discrimination real-time PCR assays identifying a single nucleotide polymorphism that was originally suggested to distinguish between neuropathogenic and non-neuro-pathogenic EHV-1 strains have been developed (Smith *et al.,* 2012). However, investigations in many countries worldwide demonstrated that the nucleotide substitution was not a reliable predictor of enhanced neuropathogenicity. Multilocus typing and whole genome sequencing are useful for molecular epidemiological studies (Garvey *et al.,* 2019; Nugent *et al.,* 2006; Sutton *et al.,* 2019).

1.3. Virus isolation

Virus isolation is no longer a routine test used for EHV-1 detection in the majority of diagnostic laboratories but is more often conducted for surveillance and research purposes. 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.). RK13 cells are commonly used for this purpose.

Recently prepared cell monolayers in tissue culture flasks or plates are inoculated with nasopharyngeal swab extract or homogenised tissue: approximately 10% (w/v) pooled tissue homogenates of liver, lung, thymus, adrenal gland and spleen (from aborted fetuses/neonatal foals) or of brain and spinal cord (from cases of neurological disease). Virus is allowed to attach by incubating the inoculated monolayers at 37°C for 1 hour after which the inocula are removed and the monolayers are rinsed twice with PBS or maintenance medium. Monolayers of uninoculated control cells should be incubated in parallel. 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 in a 5% CO_2 environment.

The use of a positive control of relatively low titre may be used to validate the isolation procedure but should be processed separately to avoid contamination of diagnostic specimens. 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.

Blood samples: EHV-1 can be isolated from PBMC. Buffy coats may be prepared from unclotted (heparinised) blood by centrifugation at 525 g for 5 minutes. The buffy coat is taken after the plasma has been carefully removed and washed three times in 3 ml MEM containing 2% FCS (525 g for 5 minutes). Following the third wash, the buffy coat is harvested and resuspended in 2.5 ml MEM containing 2% FCS. An aliquot of the rinsed cell suspension can be used for DNA extraction. For virus isolation, the resuspended cells (1 ml) are co-cultivated with freshly prepared primary equine lung or RK-13 cell suspensions (5 ml) in 25 cm² flasks. Confluent cell monolayers are not used. The flasks are incubated at

37°C in a 5% CO_2 environment for 3 days or until the cells have reached 90% confluence. The monolayers are then rinsed three times with 1 × PBS and supplemented with 5 ml MEM containing 2% FCS. They are incubated for a further 4 days and observed daily for viral CPE. 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 a WOAH Reference Laboratory for strain characterisation and to maintain a geographically diverse archive.

1.4. Virus detection by direct immunofluorescence

Direct immunofluorescent detection of EHV-1 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), 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 frozen or fixed 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 may also be carried out on infected cell monolayers. Adequate controls must be included with each immunoperoxidase test run for evaluation of both the method specificity and antibody specificity. If non-specific polyclonal sera are used the staining method needs to be combined with virus isolation or PCR to discriminate between EHV-1 and EHV-4.

1.6. Histopathology

Histopathological examination of sections of fixed placenta and lung, liver, spleen, adrenal gland 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.

Serum antibody levels to EHV-1/4 may be determined by virus neutralisation (VN) (Thomson *et al*., 1976), complement fixation tests (CFT) (Thomson *et al*., 1976) or enzyme-linked immunosorbent assay (ELISA) (Crabb & Studdert, 1995). 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. Commercial ELISAs that distinguish EHV-1 and EHV-4 antibodies are available but less widely used than the CF and VN tests. An ELISA using a synthetic peptide for glycoprotein E as an antigen (Andoh *et al.,* 2013) is used as DIVA[1] for horses vaccinated with a modified live EHV-1 vaccine licensed in Japan, that lacks the glycoprotein E gene.

The VN and the CF tests are widely used and sensitive serological assays 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 three replicate wells for each serum dilution are required. Heat-inactivated maintenance medium with a concentration of 2% FCS (HIMM) 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 RK-13 cells are prepared. 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 ≥75% of the cell monolayer from virus destruction in 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) Prepare semi-confluent monolayers in tissue culture microtitre plates.

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

iii) Add 40 µl of HIMM to all wells of the microtitre assay plates.

iv) For test sample titration, pipette 40 µl of each test serum into triplicate wells of the plate. The first two rows serve as the dilution of the test serum and the third row serves as the serum toxicity control. Make doubling dilutions of each serum by sequential mixing and transfer of 40 µl to each subsequent row of wells. Add 40µl of HIMM to the serum control rows.

v) Add 40 µl of the appropriately diluted EHV-1 virus stock to all wells (100 $TCID_{50}$/well) of the test plate except those of the serum controls. Note that the final serum dilutions, after addition of virus, run from a starting dilution of 1/4. A separate control plate should include titration of both a negative and positive (high and low) horse sera of known titre, cell control

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1   DIVA: detection of infection in vaccinated animals

vi) (no virus), and a back titration of virus using six wells per log dilution (100 $TCID_{50}$ to 0.01 $TCID_{50}$/well).

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

vii) Transfer 50 µl from each well of the test and control plates to the tissue culture microtitre plates.

viii) Incubate the plates for 2–5 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. Confirm the validity of the test by establishing that the working dilution of stock virus is at 100 $TCID_{50}$/well, that the (high and low) positive control sera are within one well of their pre-determined titre and that the negative control serum is negative at a 1/4 dilution. This takes approximately 72 hours. If at this stage the antigen is too weak the virus concentration may be increased by extending the incubation period up to 5 days. If the antigen is too strong the test must be repeated.

Wells are scored as positive for neutralisation of virus if $\geq$ 75% of the cell monolayer remains intact. The highest dilution of serum resulting in $\geq$ 75% neutralisation of virus (<25% CPE) in replicate wells is the end-point titre for that serum. Examine the plates microscopically for CPE and record the results on a worksheet.

x) 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. Wells containing intact cell monolayers stain blue, while monolayers destroyed by virus do not stain.

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.

2.2. Complement fixation test

The CFT can be used for the detection and quantification of antibodies to EHV-1. The test determines whether an antigen and an antibody are capable of forming a complex. The presence of an immune complex is revealed by the detector system, which consists of guinea-pig complement and sensitised sheep red blood cells (SRBCs) coated with rabbit haemolytic serum (haemolysin). In the absence of antibodies against equine herpesvirus, no antibody/antigen complex is formed, the complement remains free in the solution and the sensitised SRBCs become lysed. In the presence of antibodies against equine herpesvirus, an antibody/antigen complex is formed, the complement becomes fixed and is therefore unable to lyse the SRBCs. They subsequently form a button at the bottom of the test well.

Guinea-pig complement, rabbit haemolytic serum, complement fixation diluent (CFD) and bovine serum albumin (BSA) can be obtained commercially. The dilution of guinea-pig complement that has activity at 3 HD (haemolytic dose) in the presence of sensitised SRBCs should be optimised. The recommended dilution of rabbit haemolytic serum (or the working dilution) is sometimes provided by the supplier. However, the optimal dilution of haemolysin should be determined with the in use reagents (complement etc.) so that the test can be performed reproducibly. The optimum concentration of antigen to be used in the test should be determined using an antigen versus antibody chequerboard technique and by testing a panel of known positive sera.

The test is performed in U bottomed microtitre plates. Paired sera should be assayed on the same plate. An antibody positive serum should be included as a control on each plate. All sera are tested on a second plate containing all components except virus to check for anti-complementary activity. A back titration of the working dilution (3 HD) of complement to 2 HD, 1 HD, 0.5 HD is set up in duplicate wells on the complement control plate (eight wells in total). An SRBC control is set up in eight wells.

2.2.3. Preparation of samples

i) Samples and controls are prepared by adding 4 volumes (600 µl) of CFD to 1 volume (150 µl) of test sera to give a 1/5 dilution.

ii)   Diluted serum is inactivated for 30 minutes at 60°C to destroy the naturally occurring complement.

2.2.4. Test procedure

i)   Prepare the test plate and anti-complementary plate by adding 25 µl 0.05% BSA/CFD to all wells except the first column (H).

ii)   Add 50 µl of 0.05% BSA/CFD to the eight wells of the complement control (back titration).

iii)   Add 75 µl of 0.05% BSA/CFD to eight wells of cell control.

iv)   Add 50 µl of the diluted inactivated test serum and controls to the first well of each row on both the test and anti-complementary plates. Serial doubling dilutions are then made by transferring 25 µl across the plate and discarding the final 25 µl.

v)   Place the microtitre plates on ice for addition of antigen and complement.

vi)   Add 25 µl of antigen (diluted to working strength in 0.05% BSA/CFD) to the test plates.

vii)   Add 25 µl of 0.05% BSA/CFD to all wells of the anti-complementary plate to compensate for lack of antigen.

viii)   Add 25 µl of guinea-pig complement diluted in 0.05% BSA/CFD to 3 HD to all wells except the complement control and SRBC control.

ix)   Back titrate the working dilution of 3 HD complement to 2 HD, 1 HD and 0.5 HD in 200 µl volumes. Add 25 µl of each dilution to the appropriate wells.

x)   Incubate all plates at 4°C overnight.

2.2.5. Preparation and addition of sheep blood

i)   SRBCs collected into Alsever's solution are washed twice in 0.05% BSA/PBS solution.

ii)   Gently resuspend the SRBCs in 5–10 ml 0.05% BSA/CFD solution. Dilute to 2% SRBCS (v/v packed cells) in BSA/CFD solution.

iii)   Mix the 2% SRBCs with an equal volume of BSA/CFD solution containing haemolysin at its optimal sensitising concentration to give a 1% SRBC solution. Prepare an appropriate volume of this solution by allowing 3 ml per microtitre plate.

iv)   Incubate at 37°C for 10 minutes. Store the 1% sensitised SRBCs overnight at 4°C.

v)   The following day, incubate the 1% sensitised SRBCs at 37°C for 30 minutes. During the final 20 minutes of this incubation, transfer the test plates from 4°C to 37°C.

vi)   At the end of the 30-minute incubation, add 25 µl of 1% sensitised SRBCs to all plates. Mix on a plate shaker for 30 seconds.

vii)   Incubate the plates at 37°C for 30 minutes. Shake the plates after 15 minutes and at the end of this incubation (a total of three times).

viii)   Incubate the plates at 4°C for 2 hours to allow the cells to settle.

ix)   Read and record the test results after 2 hours.

2.2.6. Reading results

i)   Confirm the validity of the test by establishing that the working dilution of complement is at 3 HD: 100% lysis at 3 HD and 2 HD, and 50% lysis at 1 HD. Distinct buttons should be visible in the eight wells of the SRBC control.

ii)   There must be 100% lysis observed at the 1/5 dilution for the negative control (<5). The antibody titre of the positive control serum must read within one well of its predetermined titre.

iii)   Confirm that there are no buttons visible on the anti-complementary plates. Buttoning indicates either the presence of residual native complement in the sample or that there is a

non-specific complement fixing effect occurring. Sera that show anti-complementary activity should be retested and treated as described below.

iv) In the test wells, buttoning indicates the presence of antibodies in the serum. The antibody titre is the dilution at which there is 50% buttoning and 50% lysis observed.

2.2.7. Treatment of samples showing anti-complementary activity

i) Add 50 µl of guinea-pig complement to 150 µl of the serum showing anti-complementary activity.

ii) Incubate the sample at 37°C for 30 minutes.

iii) Add 550 µl of CFD (1:5 dilution).

iv) Heat inactivate at 60°C for 30 minutes.

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 protect 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. A minority of 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 have 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. 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 (< 1:24 by VN test) to EHV-1/4, with doses of the test vaccine that will be recommended on the final product label (Goodman *et al.,* 2006; Van de Walle *et al.,* 2010). 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. Although potency testing on production batches of ER vaccine may be performed by vaccination of susceptible horses followed by assay for seroconversion, rapid *in-vitro* immunoassays exist for antigenic potency. The basis for such *in-vitro* assays for ER vaccine potency is the determination, by use of 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 achieved by immunisation with each batch of vaccine are not required. The results of many reported observations indicate that immunity induced by vaccination-against EHV-1 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 WOAH Reference Laboratories for equine rhinopneumonitis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH 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 2024.

Appendix 1: Equine rhinopneumonitis (infection with Varicellovirus equidalpha1)
Intended purpose of test: population freedom from infection

Test with score and species	Test populations	Validation report	Advantages	Disadvantages	References
VN ++ IgG	20 foals for vaccine study 15 ponies for experimental infection 163 horses 86 mules 128 donkeys	No robust diagnostic validation studies have been published	Antibodies are durable VN evaluates status over longer period than virus detection methods or CFT	Does not differentiate between EHV-1 and EHV-4 Requires live virus and cell culture Latently infected horse may not be seropositive	Heldens *et al.,* (2001 Hussey *et al.,* (2006 El Brini *et al.,* (2021 Ataseven *et al.,* (2009
ELISA + Glycoprotein G	163 horses 86 mules 128 donkeys 237 mares 200 foals	No robust diagnostic validation studies have been published	Easier to perform than VN Can upscale readily for testing large numbers of samples	Less sensitive for EHV-1 than VN Latently infected horse may not be seropositive	El Brini *et al.,* 2021 Ataseven *et al.,* 2009 Foote *et al.,* 2003 Gilkerson *et al.,* 1999

**Appendix 2: Equine rhinopneumonitis (infection with Varicellovirus equidalpha1)
Intended purpose of test: individual freedom from infection prior to movement**

Test with score and species	Test populations	Validation report	Advantages	Disadvantages	References
PCR +++ DNA	231 healthy performance horses 162 healthy sport horses 167 imported horses in quarantine	No robust diagnostic validation studies have been published	Highly sensitive and specific Rapid	"Snapshot in time" i.e. dependent on the status of the animal at a specific time point when the sample is collected Risk of contamination in the laboratory or at sample collection by the clinician may result in false positives due to sensitivity of test Positivity does not necessarily equate with infectious virus and risk to cohort	Pusterla *et al.*, 2023 Pusterla *et al.*, 2022 Smith *et al.*, 2018
Virus isolation ++ Infectious virus	15 experimentally infected ponies tested by virus isolation and PCR 327 foals in epidemiological survey tested by virus isolation and conventional PCR	No robust diagnostic validation studies have been published	Unequivocal -detects infectious virus	Less sensitive than PCR Slow – time consuming Requires tissue culture facilities Dependent on the status of the animal at a specific time point when the sample is collected	Hussey *et al.*, 2006 Lawrence *et al.*, 1994
CFT ++ IgG and IgM	33 acute and convalescent serum samples from experimentally or naturally infected horses.	No robust diagnostic validation studies have been published	Enables a diagnosis of recent infection to be reached when virus detection is negative. The comparison of paired samples collected 14–28 days apart covers a far longer period than a sample collected for virus detection	Paired samples collected 14–28 days apart required	Hartley *et al.*, 2005
VN ++	33 acute and convalescent serum samples from experimentally or naturally infected horses.	No robust diagnostic validation studies have been published	As for CFT (see above)	Paired samples collected 14–28 days apart required	Hartley *et al.*, 2005
ELISA++	33 acute and convalescent serum samples from experimentally or naturally infected horses.	No robust diagnostic validation studies have been published	As for CFT (see above)	Paired samples collected 14–28 days apart required	Hartley *et al.*, 2005

Appendix 3: Equine rhinopneumonitis (infection with Varicellovirus equidalpha1)
Intended purpose of test: confirmation of clinical cases

Test with score and species	Test populations	Validation report	Advantages	Disadvantages	References
PCR +++ DNA	Investigation of outbreak with 46 neurological cases 60 horses during neurological outbreak 244 abortions Abortion outbreak (18 fetuses) Abortion outbreak (7 abortions and 1 neonatal foal death)	No robust diagnostic validation studies have been published	Highly sensitive and specific Rapid	Risk of contamination in the laboratory or at sample collection by the clinician may result in false positives due to sensitivity of test	Henninger *et al.,* 2007 Couroucé *et al.,* 2023 Garvey *et al.,* 2019 Barbic *et al.,* 2012 Van Maanen *et al.,* 2000
Virus Isolation ++ Infectious virus	Abortion outbreak (7 abortions and 1 neonatal foal death) Investigation of outbreak with 46 neurological cases Investigation of EHV-1 paresis and neonatal foal disease (170 animals) Neurological outbreak (13 premises) Abortion outbreak (18 fetuses)	No robust diagnostic validation studies have been published	Unequivocal -detects infectious virus	Less sensitive than PCR Slow – time consuming Requires tissue culture facilities	Van Maanen *et al.,* 2000 Henninger *et al.,* 2007 McCartan *et al.,* 1995 Gryspeerdt *et al.,* 2011 Barbic *et al.,* 2012
Direct immunofluorescence ++ Antigens	Abortion outbreak (7 abortions and 1 neonatal foal death)	No robust diagnostic validation studies have been published	Rapid preliminary diagnosis in tissues	Less sensitive than PCR	Van Maanen *et al.,* 2000 Gunn *et al.,* 1992
CFT ++ IgG and IgM	Investigation of EHV-1 paresis and neonatal foal disease (170 horses) Investigation of EHV1 neurological outbreak 122 horses Abortion outbreak (7 abortions and 1 neonatal foal death)	No robust diagnostic validation studies have been published	Enables a diagnosis of recent infection to be reached when virus detection is negative. The comparison of paired samples collected 14–28 days apart covers a far longer period than a sample collected for virus detection Single samples may be useful as CF antibodies usually decline quickly and high titres may support, if not confirm a diagnosis	Does not differentiate between EHV-1 and -4 Paired samples collected 14–28 days apart required for confirmatory diagnosis and thus, may be retrospective Poor humoral response reported in viraemic foals	McCartan *et al.,* 1995 Strang & Newton (2017 Van Maanen *et al.,* 2000

Test with score and species	Test populations	Validation report	Advantages	Disadvantages	References
VN ++ IgG	Investigation of outbreak with 46 neurological cases Abortion outbreak Abortion outbreak (7 abortions and 1 neonatal foal death)	No robust diagnostic validation studies have been published	Enables a diagnosis of recent infection to be reached when virus detection is negative The comparison of paired samples collected 14–28 days apart covers a far longer period than a sample collected for virus detection	Paired samples collected 14–28 days apart required. Retrospective or delayed diagnosis. Does not differentiate between EHV-1 and -4	Henninger *et al.,* 2007 Gryspeerdt *et al.,* 2011 Van Maanen *et al.,* 2000
ELISA ++	Abortion outbreak (7 abortions and 1 neonatal foal death) 33 acute and convalescent serum samples from experimentally or naturally infected horses	No robust diagnostic validation studies have been published	Easier to perform than VN or CFT Can upscale readily for testing large numbers of samples. Differentiates EHV-1 and -4	More validation required in the field	Van Maanen *et al.,* 2000 Hartley *et al.,* 2005

**Appendix 4: Equine rhinopneumonitis (infection with Varicellovirus equidalpha1)
Intended purpose of test: prevalence of infection – surveillance**

Test with score and species	Test populations	Validation report	Advantages	Disadvantages	References
VN +++ IgG	163 horses 53.6% (37/69) EHV1 (ELISA negative were positive by VN and 90.5%. ELISA positives were positive by VN 86 mules 128 donkeys	No robust diagnostic validation studies have been published	Antibodies are durable More sensitive than ELISA	Requires tissue culture facilities and live virus Labour intensive Does not differentiate between EHV-1 and EHV-4	El Brini *et al.*, 2021 Ataseven *et al.*, 2009
ELISA ++	163 horses 290 horses 237 mares 200 foals	No robust diagnostic validation studies have been published	Easier to perform than VN Can upscale readily for testing large numbers of samples Type-specific ELISA may differentiate EHV-1 and -4	Less sensitive than VN for EHV-1 Almost 100% samples test positive for EHV-4 More field validation required	El Brini *et al.*, 2021 Ataseven *et al.*, 2009 Foote *et al.*, 2004 Gilkerson *et al.*, 1999

**Appendix 4: Equine rhinopneumonitis (infection with Varicellovirus equidalpha1)
Intended purpose of test: immune status in individual animals or populations
(post-vaccination)**

Test with score and species	Test populations	Validation report	Advantages	Disadvantages	References
VN +++ IgG	Vaccinated mares 192 modified live vaccine and 150 inactivated vaccine 50 racehorses 30 racehorses 20 foals	No robust diagnostic validation studies have been published	Antibodies are durable Some evidence of correlation with duration of virus excretion	Requires tissue culture facilities and live virus Labour intensive Does not differentiate between EHV-1 and EHV-4	Bresgen *et al.,* 2012 Bannai *et al.,* 2019 Gildea *et al.,* 2016 Heldens *et al.,* 2001
CFT +++ IgG and IgM	71 weanlings 20 foals and 5 pregnant mares 5 pregnant mares 9 adult horses	No robust diagnostic validation studies have been published	Antibodies may be detectable earlier than VN antibodies post-vaccination Some evidence of correlation with decreased clinical signs	Antibodies are less durable than VN antibodies Does not differentiate between EHV-1 and EHV-4	Allkofer *et al.,* 2021 Heldens *et al.,* 2001 Kydd *et al.,* 2003 Singh *et al.,* 2004
ELISA ++ Glycoprotein G	44 racehorses 106 racehorses	No robust diagnostic validation studies have been published	Easier to perform than VN or CFT Can upscale readily for large numbers of samples Type-specific ELISA may differentiate EHV-1 and -4	Some evidence that ELISA is less sensitive than CFT or VN for measurement of immune response to vaccination	Yasunaga *et al.,* 2000 Bannai *et al.,* 2014