* 1. **Annex 13. Item 5.1. – Chapter 3.6.9. Equine rhinopneumonitis (infection with equid herpesvirus-1)**
  2. MEETING OF THE BIOLOGICAL STANDARDS COMMISSION
  3. **Paris, 4–8 September 2023**

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6 **CHAP T E R 3 . 6 . 9 .**

# 7 E QU I N E R H IN OP N E U M ON I T IS ( I N FE CT I ON WIT H

8 **EQU I D H ERPESVI RU S - 1  ~~AN D - 4~~ )**

9 **SUMMARY**

1. *Equine rhinopneumonitis* (*ER*) *is a collective term for any one of several contagious, clinical disease entities*
2. *of equids that may occur as a result of infection by either of* ***t****wo closely related herpesviruses, equid*
3. *alphaherpesvirus-1 and -4* (*EHV-1 and EHV-4*)*. Infection with EHV-1 is listed by WOAH and is therefore the*
4. *focus of this chapter. EHV-1 is ~~and EHV-4 are~~ endemic in most domestic equine populations worldwide.*
5. *Primary infection by ~~either~~ EHV-1 ~~or EHV-4~~ is characterised by upper respiratory tract disease of varying*
6. *severity that is related to the age and immunological status of the infected animal. Following viraemia EHV-*
7. *1 also causes the more serious complications of abortion, perinatal foal death, or paralytic neurological*
8. *disease* (*equine herpesvirus myeloencephalopathy*)*. ~~EHV-4 has been associated with sporadic cases of~~*
9. *~~abortion, but rarely multiple abortions and not the large outbreaks associated with EHV-1.~~ Like other*
10. *herpesviruses, EHV-1 ~~and 4~~ induces long-lasting latent infections and can be reactivated following stress ~~or~~*
11. *~~pregnancy~~. Furthermore, most horses are likely to be re-infected multiple times during their lifetime, often*
12. *~~mildly or~~ subclinically. ~~Detection of viral DNA or anti-EHV antibodies should therefore be interpreted with~~*
13. *~~care.~~*
14. ***Identification of the agent:*** *The standard method of identification of EHV-1 ~~and EHV-4~~ from appropriate*
15. *clinical or necropsy material is by polymerase chain reaction* (*PCR*)*~~, followed by laboratory isolation of the~~*
16. *~~virus in cell culture~~.*
17. *Positive identification of viral isolates as EHV-1 ~~or EHV-4~~ can be achieved by type-specific PCR or*
18. *sequencing. Viruses can be isolated in ~~equine~~ cell culture from nasal or nasopharyngeal swab extracts taken*
19. *from horses ~~during the febrile stage of~~ with acute respiratory tract infection, from the placenta, from ~~and~~ liver,*
20. *lung, spleen, adrenal glands or thymus of aborted fetuses and early foal deaths, and from the leukocyte*
21. *fraction of the blood of animals ~~with acute~~ during the febrile stage of EHV-1 infection. ~~Unlike EHV-4, EHV-1~~*
22. *~~will also grow in various non-equine cell types such as the RK-13 cell line and this property can be used to~~*
23. *~~distinguish between the two viruses.~~*
24. *A rapid presumptive diagnosis of abortion induced by EHV-1 ~~or~~* ~~(~~*~~infrequently~~*~~)~~ *~~EHV-4~~ can be achieved by*
25. *direct immunofluorescent detection of viral antigen in cryostat sections of placenta and tissues from aborted*
26. *fetuses, using a conjugated polyclonal antiserum.*
27. *Post-mortem demonstration of histopathological lesions of EHV-1 in placenta and tissues from aborted*
28. *fetuses, cases of perinatal foal death or in the central nervous system of neurologically affected animals*
29. *complements other diagnostic techniques ~~the laboratory diagnosis~~.*
30. ***Serological tests:*** *Most horses possess some level of antibody to EHV-1/4, the demonstration of specific*
31. *antibody in the serum collected from a single blood sample is therefore not confirmation of a positive*
32. *diagnosis of recent infection. Paired, acute and convalescent sera from animals suspected of being infected*
33. *with EHV-1 or EHV-4 should be tested for a four-fold or greater rise in virus-specific antibody titre by either*
34. *virus neutralisation* (*VN*) *or complement fixation* (*CF*) tests. *Neither of these assays is type-specific but both*
35. *have proven useful for diagnostic purposes especially since the CF antibody response to recent infection is*
36. *relatively short-lived. Limited use has also been made of a type-specific enzyme-linked immunosorbent*
37. *assay* ~~(~~*~~Crabb~~* ~~et al.,~~ *~~1995; Hartley~~* ~~et al.,~~ *~~2005~~*~~)~~*.*
38. ***Requirements for vaccines:*** *Both live attenuated and inactivated viral vaccines are available for use in*
39. *assisting in the control of EHV-1~~/4~~. Vaccination is helpful in reducing the severity of respiratory infection in*
40. *young horses and the incidence of abortion in mares, however current vaccines are not licenced to protect*
41. *against neurological disease. Vaccination should not be considered a substitute for sound management*
42. *practices known to reduce the risk of infection. Revaccination at frequent intervals is recommended in the*
43. *case of each of the products, as the duration of vaccine-induced immunity is relatively short.*
44. *Standards for production and licensing of both attenuated and inactivated EHV-1~~/4~~ vaccines are established*
45. *by appropriate veterinary regulatory agencies in the countries of vaccine manufacture and use. A single set*
46. *of internationally recognised standards for EHV vaccines is not available. In each case, however, vaccine*
47. *production is based on the system of a detailed outline of production employing a well characterised cell line*
48. *and a master seed lot of vaccine virus that has been validated with respect to virus identity, safety, virological*
49. *purity, immunogenicity and the absence of extraneous microbial agents.*

## 59 A. INTRODUCTION

1. Equine rhinopneumonitis (ER) is a historically-derived term that describes a constellation of several disease entities of
2. horses that may include respiratory disease, abortion, neonatal foal pneumonitis, or myeloencephalopathy (~~Allen & Bryans,~~
3. ~~1986;~~ Allen *et al*., 1999~~; Bryans & Allen, 1988; Crabb & Studdert, 1995~~). The disease ~~has been~~ is recognised ~~for over 60~~
4. ~~years~~ as a threat to the international horse industry, and is caused by either of two members of the *Herpesviridae* family,
5. equid alphaherpesvirus-1 and -4 (EHV-1 and EHV-4). EHV-1 and EHV-4 are closely related alphaherpesviruses of horses
6. with nucleotide sequence identity within individual homologous genes ranging from 55% to 84%, and amino acid sequence
7. identity from 55% to 96% (Telford *et al*., ~~1992;~~ 1998). ~~The two herpesviruses~~ With the exception of EHV-1 in Iceland
8. (Thorsteinsdóttir *et al*., 2021), the two herpesviruses are considered endemic ~~enzootic~~ in all countries in which large
9. populations of horses are maintained as part of the cultural tradition or agricultural economy. There is no recorded evidence
10. that the two herpesviruses of ER pose any health risks to humans working with the agents. Infection with EHV-1 is listed
11. by WOAH and is therefore the focus of this chapter.
12. Viral transmission to cohort animals occurs by inhalation of aerosols of virus-laden respiratory secretions. Morbidity tends
13. to be highest in young horses sharing the same air space. Aborted tissues and placental fluids from infected mares can
14. contain extremely high levels of live virus and represent a major source of infection. Extensive use of vaccines has not
15. eliminated EHV-1 infections, and the world-wide ~~annual~~ financial impact from this ~~these~~ equine pathogen~~s~~ is ~~immense~~
16. considerable.
17. In horses under 3 years of age, clinical ER usually takes the form of an acute, febrile respiratory illness that spreads rapidly
18. through the group of animals. The virus~~es~~ infects and multiplies ~~multiply~~ in epithelial cells of the respiratory mucosa. Signs
19. of infection become apparent 2–8 days after exposure to virus, and are characterised by fever, inappetence, depression,
20. and nasal discharge. The severity of respiratory disease varies with the age of the horse and the level of immunity resulting
21. from previous vaccination or natural exposure. Bi-phasic fever, viraemia and complications are more likely with EHV-1 than
22. EHV-4. Subclinical infections with EHV-1~~/4~~ are common, even in young animals. Although mortality from uncomplicated
23. ER is rare and complete recovery within 1–2 weeks is the normal outcome, respiratory infection is a frequent and significant
24. cause of interrupted schedules among horses assembled for training, racing, or other equestrian events. Fully protective
25. immunity resulting from infection is of short duration, and convalescent animals are susceptible to reinfection by EHV-1~~/4~~
26. after several months. Although reinfections ~~by the two herpesviruses~~ cause less severe or clinically inapparent respiratory
27. disease, the risks of subsequent abortion or neurological disease remain. Like other herpesviruses, EHV-1~~/4~~ causes long-
28. lasting latent infections and latently infected horses represent a potential infection risk for other horses. Virus can be
29. reactivated as a result of stress ~~or pregnancy~~. The greatest clinical threats to individual breeding, racing, or pleasure horse
30. operations posed by ER are the potential abortigenic and neurological *sequelae* of EHV-1 respiratory infection. ER
31. abortions occur annually in horse populations worldwide and may be sporadic or multiple. Foals infected *in utero* may be
32. born alive and die within a few days of birth. EHV-1 neurological disease is less common than abortions but has been
33. recorded all over the world with associated fatalities. Outbreaks result in movement restrictions and, sometimes,
34. cancellation of equestrian events (Couroucé *et al*., 2023; FEI, 2021).
35. ~~Neurological disease, also known as equine herpesvirus myeloencephalopathy, remains an infrequent but serious~~
36. ~~complication of EHV-1 infection. A single mutation in the DNA polymerase gene (ORF30) has been associated with~~
37. increased risk of neurological disease, however strains without this change can also cause paralysis (Goodman *et al*.,
38. ~~2007; Nugent~~ *~~et al.,~~* ~~2006). Strain typing techniques have been employed to identify viruses carrying the neuropathic~~
39. ~~marker, and it can be helpful to be aware of an increased risk of neurological complications. However, for practical purposes~~
40. ~~strain-typing is not relevant for agent identification, or international trade. Strain typing may be beneficial for implementation~~
41. ~~of biosecurity measures in the management of outbreaks of equine herpesvirus myeloencephalopathy.~~
42. Strain typing has been shown to be not reliable for predicting the clinical outcome of EHV-1 infection but can be useful in
43. epidemiological investigations (Garvey *et al*., 2019; Nugent *et al*., 2006; Sutton *et al*., 2019).

## 103 B. DIAGNOSTIC TECHNIQUES

1. ~~Both~~ EHV-1 ~~and EHV-4~~ is transmitted by the respiratory route and has ~~have~~ the potential to be highly contagious ~~viruses~~
2. particularly where large numbers of horses are housed in the same air space. EHV1 ~~and the former~~ can cause explosive
3. outbreaks of abortion or neurological disease. Rapid diagnostic methods are therefore essential ~~useful~~ for managing the
4. disease. Real-time polymerase chain reaction (PCR) assays are ~~widely~~ routinely used by diagnostic laboratories worldwide
5. and are both rapid and sensitive. Real-time PCR assays that allow ~~simultaneous~~ testing for EHV-1 ~~and EHV-4~~ and
6. quantification of viral load have been developed. Virus isolation has been replaced by real-time PCR as the frontline
7. diagnostic test in the majority of laboratories but can also be useful, ~~particularly for the detection of viraemia. This is also~~
8. ~~true of~~ for EHV-1 associated abortions and neonatal foal deaths, when the high level of virus in the tissues usually produces
9. a cytopathic effect in 1–3 days. Immunohistochemical or immunofluorescent approaches are employed in some
10. laboratories ~~can be extremely useful~~ for rapid diagnosis of EHV-induced abortion from fresh or embedded tissue ~~and are~~
11. ~~relatively straightforward~~. ~~Several other techniques based on enzyme-linked immunosorbent assay (ELISA) or nucleic acid~~
12. ~~hybridisation probes have also been described, however their use is often restricted to specialised laboratories and they~~
13. ~~are not included here.~~ Virus neutralisation (VN) and complement fixation (CF) are the most frequently used serological
14. tests, and seroconversion in paired samples is considered indicative of exposure to virus by natural infection or by
15. vaccination.
16. ***Table 1.*** *Test methods available for the diagnosis of equine rhinopneumonitis and their purpose*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Method** | **Purpose** | | | | | |
| [Population](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex13_3.06.09_Table_1a_pop_freedom_from_infection.pdf) [freedom](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex13_3.06.09_Table_1a_pop_freedom_from_infection.pdf) [from](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex13_3.06.09_Table_1a_pop_freedom_from_infection.pdf) [infection](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex13_3.06.09_Table_1a_pop_freedom_from_infection.pdf) | [Individual](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex13_3.06.09_Table_1b_individual_freedom_to_move.pdf) [animal](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex13_3.06.09_Table_1b_individual_freedom_to_move.pdf) [freedom from](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex13_3.06.09_Table_1b_individual_freedom_to_move.pdf) [infection prior](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex13_3.06.09_Table_1b_individual_freedom_to_move.pdf) [to movement](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex13_3.06.09_Table_1b_individual_freedom_to_move.pdf) | Contribute to eradication policies(a) | [Confirmation](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex13_3.06.09_Table_1d_confirm_clinical_case.pdf) [of clinical](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex13_3.06.09_Table_1d_confirm_clinical_case.pdf) [cases](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex13_3.06.09_Table_1d_confirm_clinical_case.pdf) | [Prevalence of](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex13_3.06.09_Table_1e_prevalence_for_surveillance.pdf) [infection -](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex13_3.06.09_Table_1e_prevalence_for_surveillance.pdf) [surveillance](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex13_3.06.09_Table_1e_prevalence_for_surveillance.pdf) | [Immune status](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex13_3.06.09_Table_1f_immune_status_individuals.pdf) [in individual](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex13_3.06.09_Table_1f_immune_status_individuals.pdf) [animals or](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex13_3.06.09_Table_1f_immune_status_individuals.pdf) [populations](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex13_3.06.09_Table_1f_immune_status_individuals.pdf) [post-vaccination](https://oieoffice365.sharepoint.com/sites/ScienceDepartment/Documents%20partages/Admin/G_SCIENCE/TERRESTRIAL%20MANUAL%20ENGLISH/Manual%202024/Chapters%20after%20BSC1/Annex13_3.06.09_Table_1f_immune_status_individuals.pdf) |
| **Identification of the agent(b)** | | | | | | |
| **Virus isolation** | – | ++ ~~+~~ | – | +++ | – | – |
| **PCR** | – | +++ | – | +++ | – | – |
| **Direct immunofluorescence** | – | – | – | ++ | – | – |
| **Detection of immune response** | | | | | | |
| **VN** | ++ | ++ | – ~~+~~ | +++ | +++ | +++ |
| **ELISA** | + | – ~~+~~ | – ~~+~~ | + ~~+~~ | ++ ~~+~~ | + |
| **CFT** | – | ~~–~~ ++ | – | +++ | – | ~~–~~ +++ |

1. Key: +++ = recommended for this purpose; ++ recommended but has limitations;

121 + = suitable in very limited circumstances; – = not appropriate for this purpose.

1. PCR = polymerase chain reaction; VN = virus neutralisation;
2. ELISA = enzyme-linked immunosorbent assay; CFT = complement fixation test.
3. (a)No eradication policies exist for equine rhinopneumonitis.
4. (b)A combination of agent identification methods applied on the same clinical sample is recommended.

#### 1. ~~Identification~~ Detection of the agent

##### 1.1. Collection and preparation of specimens

1. *Nasal/nasopharyngeal swabs:* swab extract can be used for DNA extraction and subsequent virus detection by PCR
2. using one of a variety of published techniques or commercially available kits (see below). Virus isolation can also be
3. attempted from the swab extracts. To increase the chances of isolating live virus, swabs are best obtained from
4. horses during the ~~very early, febrile stages~~ acute stage of the respiratory disease, and are collected via the nares by
5. sampling the area with a swab of an appropriate size and length for horses. After collection, the swab should be
6. removed and transported immediately to the virology laboratory in 3 ml of cold (not frozen) virus transport medium
7. (e.g. phosphate buffered saline [PBS] or serum-free MEM [minimal essential medium] with antibiotics). Virus
8. infectivity can be prolonged by the addition of bovine serum albumin, fetal calf serum or gelatine to 0.1% (w/v).
9. *Tissue samples:* total DNA can be extracted using a number of commercially available kits and used in PCR to detect
10. viral DNA (described below in Section B.1.2.1). Virus isolation from placenta and fetal tissues from suspect cases of
11. EHV-1 abortion is most successful when performed on aseptically collected samples of placenta, liver, lung, thymus,
12. adrenal glands and spleen. Virus may be isolated from post-mortem cases of EHV-1 neurological disease by culture
13. of samples of brain and spinal cord but such attempts to isolate virus are often unsuccessful; however, they may be
14. useful for PCR testing and pathological examination. Tissue samples should be transported to the laboratory and
15. held at 4°C until inoculated into tissue culture. Samples that cannot be processed within a few hours should be stored
16. at –70°C.
17. *Blood:* for virus detection by PCR or isolation from blood leukocytes, collect a 10–20 ml sample of blood, using an
18. aseptic technique in ~~citrate~~, heparin or EDTA [ethylene diamine tetra-acetic acid] anticoagulant. EDTA is the preferred
19. anticoagulant for PCR testing in some laboratories as heparin may inhibit DNA polymerase. The samples should be
20. transported without delay to the laboratory on ice, but not frozen.
21. *Cerebrospinal fluid:* the detection of EHV-1 DNA in cerebrospinal fluid has been reported in cases of neurological
22. disease.

##### 1.2. Virus detection by polymerase chain reaction

1. PCR has become the primary diagnostic method for the detection of EHV-1 and EHV-4 in clinical specimens, paraffin-
2. embedded archival tissue, or inoculated cell cultures (Borchers & Slater, 1993; Lawrence *et al*., 1994; O’Keefe *et al*.,
3. 1994; Varrasso *et al*., 2001). A variety of type-specific PCR primers have been designed to distinguish between the
4. presence of EHV-1 and EHV-4. The correlation between PCR and virus isolation techniques for diagnosis of EHV-1
5. ~~or EHV-4 is high (Varrasso~~ *~~et al~~*~~., 2001).~~ Diagnosis by PCR is rapid, sensitive, and does not depend on the presence
6. of infectious virus in the clinical sample. ~~For diagnosis of active infection by EHV,~~ PCR methods are routinely used
7. to detect EHV-1 DNA in nasopharyngeal swabs and tissue samples ~~most reliable with tissue samples from aborted~~
8. ~~fetuses and placental tissue or from nasopharyngeal swabs of foals and yearlings~~. They are particularly useful in
9. explosive outbreaks of abortion, respiratory or neurological disease in which ~~a~~ rapid identification and monitoring of
10. the virus spread is critical for guiding management strategies, including movement restrictions. PCR examination of
11. spinal cord and brain tissue, as well as peripheral blood mononuclear cells (PBMC), are important in seeking a
12. diagnosis on a horse with neurological signs (Pronost *et al*., 2012).
13. Several PCR assays have been published. A nested PCR procedure can be used to distinguish between EHV-1 and
14. EHV-4. A sensitive protocol suitable for clinical or pathological specimens (nasal secretions, blood leukocytes, brain
15. and spinal cord, fetal tissues, etc.) has been described by Borchers & Slater (1993). However, nested PCR methods
16. have a high risk of laboratory cross-contamination, and sensitive rapid one-step PCR tests to detect EHV-1 and EHV-
17. 4 (e.g. Lawrence *et al.,* 1994) are preferred. The WOAH Reference Laboratories use quantitative real-time PCR
18. assays such as those targeting heterologous sequences of major glycoprotein genes to distinguish between EHV-1
19. and EHV-4. A multiplex real-time PCR targeting glycoprotein B gene of EHV-1 and EHV-4 was described by Diallo
20. *et al.* (2007). PCR protocols have been developed that can differentiate between EHV-1 strains carrying the ORF30
21. neuropathogenic marker, using both restriction enzyme digestion of PCR products (Fritsche & Borchers, 2011) or by
22. quantitative real-time PCR (Allen *et al,* 2007, Smith *et al.*, 2012). Methods have also been developed to type strains
23. for epidemiological purposes, based on the ORF68 gene (Nugent *et al.,* 2006). The WOAH Reference Laboratories
24. employ in-house methods for strain typing, however these protocols have not yet been validated between different
25. laboratories at an international level.
26. Real-time (or quantitative) PCR has become the method of choice for ~~many~~ the majority of diagnostic ~~tests~~
27. laboratories and provides rapid and sensitive detection of viral DNA. Equine post-mortem tissues from newborn and
28. adult animals or equine fetal tissue from abortions (tissues containing lung, liver, spleen, thymus, adrenal gland and
29. placental tissues) can be used. For respiratory samples, equine nasopharyngeal swabs or deep nasal swabs
30. (submitted in a suitable viral transport medium), buffy coat, tracheal wash (TW) or broncho-alveolar lavage (BAL) are
31. all suitable. DNA should be extracted using an appropriate kit or robotic system.
32. There is no internationally standardised real-time PCR method for EHV-1 but Table 2 summarises the primer and
33. probe sequences for some of the most widely used assays. Type-specific PCR primers have been designed to
34. distinguish between the presence of EHV-1 and EHV-4. The optimised thermocycler times and temperatures are
35. documented in the publications cited.
36. ***Table 2.*** *Primer and probe sequences for EHV1~~/4~~ detection by real-time PCR*

|  |  |  |  |
| --- | --- | --- | --- |
| **Primer** | **Primer sequence (5’ to 3’)** | **Target** | **Reference** |
| Forward | CAT-GTC-AAC-GCA-CTC-CCA |  |  |
| Reverse | GGG-TCG-GGC-GTT-TCT-GT | EHV‐1 gB Diallo *et al.,* 2006 | |
| Probe | FAM-CCC-TAC-GCT-GCT-CC-MGB-NFQ |
| Forward | CAT-ACG-TCC-CTG-TCC-GAC-AGA-T |  |  |
| Reverse | GGTACTCGGCCTTTGACGAA | EHV‐1 gB Hussey *et al.,* 2006 | |
| Probe | FAM‐TGA-GAC-CGA-AGA-TCT-CCT-CCA-CCG-A‐BHQ1 |
| Forward | TAT-ACT-CGC-TGA-GGA-TGG-AGA-CTT-T |  |  |
| Reverse | TTG-GGG-CAA-GTT-CTA-GGT-GGT-T | EHV‐1 gB Pusterla *et al.,* 2009 | |
| Probe | 6FAM-ACA-CCT-GCC-CAC-CGC-CTA-CCG |
| Forward | GCG-GGC-TCT-GAC-AAC-ACA-A | EHV‐1 gC | ISO 17025 accredited for the detection of EHV-1 at WOAH Reference Laboratory |
| Reverse | TTG-TGG-TTT-CAT-GGG-AGT-GTG-TA |
| Probe | FAM‐TAA-CGC-AAA-CGG-TAC-AGA-A-BHQ1 |

1. \*~~This multiplex real-time PCR test has been validated to ISO 17025 and is designed for use in a 96-well format. This~~
2. ~~can be readily combined with automatic nucleic acid extraction methods. Discrimination between EHV-1 and EHV-4~~
3. is carried out by the incorporation of type-specific dual labelled probes based on methods published by Hussey *et al.*
4. ~~(2006) and Lawrence~~ *~~et al.~~* ~~(1994). To establish such a real-time PCR assay for diagnostic purposes, validation~~
5. ~~against blinded samples is required. Sensitivity and specificity for the assay should be determined against each target.~~
6. ~~Support for development of assays and appropriate sample panels can be obtained from the WOAH Reference~~
7. ~~Laboratories.~~ Reference material and sample panels for real-time PCR can be obtained from the WOAH Reference
8. Laboratories.
9.  **Point of care (POC) molecular tests**
10. Loop-mediated isothermal amplification (LAMP) assays for the detection of EHV-1 have been described
11. (Nemoto *et al*., 2011). An evaluation of a hydrolysis probe-based insulated isothermal PCR (iiPCR) assay
12. for the detection of EHV-1 showed it to have a high sensitivity and specificity compared with real-time PCR
13. (Balasuriya *et al*., 2017). However further validation of POC tests in the field is required.
14.  **Molecular characterisation**
15. Allelic discrimination real-time PCR assays identifying a single nucleotide polymorphism that was originally
16. suggested to distinguish between neuropathogenic and non-neuro-pathogenic EHV-1 strains have been
17. developed (Smith *et al.,* 2012). However, investigations in many countries worldwide demonstrated that
18. the nucleotide substitution was not a reliable predictor of enhanced neuropathogenicity. Multilocus typing
19. and whole genome sequencing are useful for molecular epidemiological studies (Garvey *et al.,* 2019;
20. Nugent *et al.,* 2006; Sutton *et al.,* 2019).

##### 1.3. Virus isolation

1. Virus isolation is no longer a routine test used for EHV-1 detection in the majority of diagnostic laboratories but is
2. more often conducted for surveillance and research purposes. A number of cell types may be used for isolation of
3. EHV-1 (e.g. rabbit kidney [RK-13 (AATC–CCL37)], baby hamster kidney [BHK-21], Madin–Darby bovine kidney
4. [MDBK], pig kidney [PK-15], etc.). RK13 cells are commonly used for this purpose. ~~For efficient primary isolation of~~
5. EHV-4 from horses with respiratory disease, equine-derived cell cultures must be used. Both EHV-1 and EHV-4 may
6. be isolated from nasopharyngeal samples using primary equine fetal kidney cells or equine fibroblasts derived from
7. dermal (E-Derm) or lung tissue. EHV-1 can be isolated on other cell types, as will be discussed later. The
8. nasopharyngeal swab and its accompanying transport medium are transferred into the barrel of a sterile 10 ml
9. syringe. Using the syringe plunger, the fluid is squeezed from the swab into a sterile tube. A portion of the expressed
10. fluid can be filtered through a sterile, 0.45 µm membrane syringe filter unit into a second sterile tube if heavy bacterial
11. contamination is expected, but this may also lower virus titre. Recently prepared cell monolayers in tissue culture
12. flasks are inoculated with the filtered, as well as the unfiltered, nasopharyngeal swab extract. Cell monolayers in
13. multiwell plates incubated in a 5% CO2 environment may also be used. Virus is allowed to attach by incubating the
14. inoculated monolayers at 37°C. Monolayers of uninoculated control cells should be incubated in parallel.
15. ~~At~~ Recently prepared cell monolayers in tissue culture flasks or plates are inoculated with nasopharyngeal swab
16. extract or homogenised tissue: approximately 10% (w/v) pooled tissue homogenates of liver, lung, thymus, adrenal
17. and spleen (from aborted fetuses/neonatal foals) or of brain and spinal cord (from cases of neurological disease).
18. Virus is allowed to attach by incubating the ~~end of the attachment period,~~ inoculated monolayers at 37°C for 1 hour
19. after which the inocula are removed and the monolayers are rinsed twice with PBS ~~to remove virus-neutralising~~
20. ~~antibody that may~~ or maintenance medium. Monolayers of uninoculated control cells should be ~~present in the~~
21. ~~nasopharyngeal secretions~~ incubated in parallel. After addition of supplemented maintenance medium (MEM
22. containing 2% fetal calf serum [FCS] and twice the standard concentrations of antibiotics/antifungals [penicillin,
23. streptomycin, gentamicin, and amphotericin B]), the flasks are incubated at 37°C in a 5% CO2 environment.
24. The use of a positive control ~~virus samples~~ of relatively low titre may be used to validate the isolation procedure
25. ~~carries the risk that this may lead~~ but should be processed separately to ~~eventual~~ avoid contamination of diagnostic
26. specimens. ~~This risk can be minimised by using routine precautions and good laboratory technique, including the use~~
27. of biosafety cabinets, inoculating positive controls after the diagnostic specimens, decontaminating the surfaces in
28. ~~the hood while the inoculum is adsorbing and using a positive control of relatively low titre.~~ Inoculated flasks should
29. be inspected daily by microscopy for the appearance of characteristic herpesvirus cytopathic effect (CPE) (focal
30. rounding, increase in refractility, and detachment of cells). Cultures exhibiting no evidence of viral CPE after 1 week
31. of incubation should be blind-passaged into freshly prepared monolayers of cells, using small aliquots of both media
32. and cells as the inoculum. Further blind passage is usually not productive.
33. It can be useful to inoculate samples into both non-equine and equine cells in parallel to distinguish between EHV-1
34. and EHV-4, since EHV-4 can cause sporadic cases of abortion. Around 10% (w/v) pooled tissue homogenates of
35. liver, lung, thymus, and spleen (from aborted fetuses) or of central nervous system tissue (from cases of neurological
36. disease) are used for virus isolation. These are prepared by first mincing small samples of tissue into 1 mm cubes in
37. a sterile Petri dish with dissecting scissors, followed by macerating the tissue cubes further in serum-free culture
38. medium with antibiotics using a homogeniser or mechanical tissue grinder. After centrifugation at 1200 ***g*** for
39. 10 minutes, the supernatant is removed and 0.5 ml is inoculated into duplicate cell monolayers in tissue culture flasks.
40. Following incubation of the inoculated cells at 37°C for 1.5–2 hours, the inocula are removed and the monolayers are
41. rinsed twice with PBS or maintenance medium. After addition of 5 ml of supplemented maintenance medium, the
42. flasks are incubated at 37°C for up to 1 week or until viral CPE is observed. Cultures exhibiting no evidence of viral
43. CPE after 1 week of incubation should be passaged a second time into freshly prepared monolayers of cells, using
44. ~~small aliquots of both media and cells as the inoculum.~~
45. Blood samples: EHV-1 ~~and, infrequently, EHV-4~~ can be isolated from PBMC. Buffy coats may be prepared from
46. unclotted (heparinised) blood by centrifugation at ~~600~~ 525 ***g*** for ~~15~~ 5 minutes~~, and~~. The buffy coat is taken after the
47. plasma has been carefully removed~~. The buffy coat is then layered onto a PBMC separating solution (Ficoll; density~~
48. 1077 g/ml, commercially available) and centrifuged at 400 ***g*** for 20 minutes. The PBMC interface (without most
49. ~~granulocytes) is~~ and washed ~~twice in PBS (300~~ ***~~g~~*** ~~for 10 minutes) and resuspended in 1 ml~~ three times in 3 ml MEM
50. containing 2% FCS~~. As a quicker alternative method, PBMC may be collected by centrifugation directly from plasma.~~
51. (525 ***g*** for 5 minutes). Following the third wash, the buffy coat is harvested and resuspended in 2.5 ml MEM containing
52. 2% FCS. An aliquot of the rinsed cell suspension ~~is added to each of the duplicate monolayers of equine fibroblast,~~
53. ~~equine fetal or RK-13 cell monolayers in 25 cm~~2 ~~flasks containing 8–10 ml freshly added maintenance medium. The~~
54. ~~flasks~~ can be used for DNA extraction. For virus isolation, the resuspended cells (1 ml) are co-cultivated with freshly
55. prepared primary equine lung or RK-13 cell suspensions (5 ml) in 25 cm2 flasks. Confluent cell monolayers are not
56. used. The flasks are incubated at 37°C in a 5% CO2 environment for 3 days or until the cells have reached 90%
57. confluence. The monolayers are then rinsed three times with 1 × PBS and supplemented with 5 ml MEM containing
58. 2% FCS. They are incubated ~~at 37°C for 7 days; either with or without removal of the inoculum. If PBMCs are not~~
59. removed prior to incubation, CPE may be difficult to detect in the presence of the massive inoculum of leukocytes:
60. ~~each flask of cells is freeze–thawed after 7~~ for a further 4 days ~~of incubation and the contents centrifuged at 300~~ ***~~g~~***
61. for 10 minutes. Finally, 0.5 ml of the cell-free, culture medium supernatant is transferred to freshly made cell
62. ~~monolayers that are just subconfluent. These are incubated~~ and observed daily for viral CPE ~~for at least 5–6 days.~~
63. ~~Again, samples~~. Samples exhibiting no evidence of viral CPE after 1 week of incubation should be passaged a second
64. time before discarding as negative.
65. Virus identity may be confirmed by PCR or by immunofluorescence with specific antisera. Virus isolates from
66. positive cultures should be submitted to a WOAH Reference Laboratory for strain characterisation and to maintain
67. a geographically diverse archive. ~~Further strain characterisation for surveillance purposes or detection of the~~
68. ~~neurological marker can be provided at some laboratories.~~

##### 1.4. Virus detection by direct immunofluorescence

1. Direct immunofluorescent detection of EHV-1 antigens in samples of post-mortem tissues collected from aborted
2. equine fetuses and the placenta provides a rapid preliminary diagnosis of herpesvirus abortion (Gunn, 1992). The
3. diagnostic reliability of this technique approaches that of virus isolation attempts from the same tissues.
4. In the United States of America (USA), potent polyclonal antiserum to EHV-1, prepared in swine and conjugated with
5. FITC, is available to veterinary diagnostic laboratories for this purpose from the National Veterinary Services
6. Laboratories of the United States Department of Agriculture (USDA). The antiserum cross-reacts with EHV-4 and
7. hence is not useful for serotyping, however virus typing can be conducted on any virus positive specimens by PCR.
8. Freshly dissected samples (5 × 5 mm pieces) of fetal tissue (lung, liver, thymus, and spleen) are frozen, sectioned
9. on a cryostat at –20°C, mounted on to microscope slides, and fixed with 100% acetone. After air-drying, the sections
10. are incubated at 37°C in a humid atmosphere for 30 minutes with an appropriate dilution of the conjugated swine
11. antibody to EHV-1. Unreacted antibody is removed by two washes in PBS, and the tissue sections are then covered
12. with aqueous mounting medium and a cover-slip, and examined for fluorescent cells indicating the presence of EHV
13. antigen. Each test should include a positive and negative control consisting of sections from known EHV-1 infected
14. and uninfected fetal tissue.

##### 1.5. Virus detection by immunoperoxidase staining

1. Immunohistochemical (IH) staining methods, such as immunoperoxidase, have been developed for detecting EHV-1
2. antigen in fixed tissues of aborted equine fetuses, placental tissues or neurologically affected horses (Schultheiss *et*
3. *al*., 1993; Whitwell *et al*., 1992). Such techniques can be used as an alternative to immunofluorescence described
4. above and can also be readily applied to archival frozen or fixed tissue samples. Immunohistochemical staining for
5. EHV-1 is particularly useful for the simultaneous evaluation of morphological lesions and the identification of the virus.
6. Immunoperoxidase staining for EHV-1~~/4~~ may also be carried out on infected cell monolayers ~~(van Maanen~~ *~~et al~~*~~.,~~
7. ~~2000)~~. Adequate controls must be included with each immunoperoxidase test run for evaluation of both the method
8. specificity and antibody specificity. ~~In one WOAH Reference Laboratory, this method is used routinely for frozen or~~
9. ~~fixed tissue, using~~ If non-specific ~~rabbit~~ polyclonal sera is used ~~raised against EHV-1. This staining method is not~~
10. ~~type-specific and therefore~~ the staining method needs to be combined with virus isolation or PCR to discriminate
11. between EHV-1 and EHV-4~~, however it provides a useful method for rapid diagnosis of EHV-induced abortion~~.

##### 1.6. Histopathology

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

#### 2. Serological tests

1. EHV-1 and EHV-4 are endemic in most parts of the world and seroprevalence is high, however serological testing of paired
2. sera can be useful for diagnosis of ER in horses. A positive diagnosis is based on the demonstration of significant increases
3. (four-fold or greater) in antibody titres in paired sera taken during the acute and convalescent stages of the disease. The
4. results of tests performed on sera from a single collection date are, in most cases, impossible to interpret with any degree
5. of confidence. The initial (acute phase) serum sample should be taken as soon as possible after the onset of clinical signs,
6. and the second (convalescent phase) serum sample should be taken 2–4 weeks later.
7. ‘Acute phase’ sera from mares after abortion or from horses with EHV-1 neurological disease may already contain maximal
8. titres of EHV-1 antibody, with no increase in titres detectable in sera collected at later dates. In such cases, serological
9. testing of paired serum samples from clinically unaffected cohort members of the herd may prove useful for retrospective
10. diagnosis of ER within the herd.
11. ~~Finally, the serological detection of antibodies to EHV-1 in heart or umbilical cord blood or other fluids of equine fetuses~~
12. ~~can be of diagnostic value in cases of abortion especially when the fetus is virologically negative. The EHV 1/4 nucleic acid~~
13. ~~may be identified from these tissues by PCR.~~
14. Serum antibody levels to EHV-1/4 may be determined by virus neutralisation (VN) (Thomson *et al*., 1976), complement
15. fixation (CF) tests (Thomson *et al*., 1976) or enzyme-linked immunosorbent assay (ELISA) (Crabb & Studdert, 1995). ~~There~~
16. ~~are no internationally recognised reagents or standardised techniques for performing any of the serological tests for~~
17. ~~detection of EHV-1/4 antibody; titre determinations on the same serum may differ from one laboratory to another.~~
18. ~~Furthermore,~~ The CF and VN tests detect antibodies that are cross-reactive between EHV-1 and EHV-4. Nonetheless, the
19. 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
20. serological confirmation of recent infection with one of the viruses. Commercial ELISAs that distinguish EHV-1 and EHV-
21. 4 antibodies are available but less widely used than the CF and VN tests. Unlike other alphaherpesviruses, DIVA [41](#_bookmark123) ELISAs,
22. which have been very useful in eradication programmes for bovine rhinotracheitis and pseudorabies (Aujeszky’s disease),
23. have not been developed for EHV-1/4 .
24. The ~~microneutralisation test is a~~ VN and the CF tests are widely used and sensitive serological assays for detecting EHV-
25. 1/4 antibody and will thus be described here.

##### 2.1. Virus neutralisation test

1. This test is most commonly performed in flat-bottom 96-well microtitre plates (tissue culture grade) using a constant
2. dose of virus and doubling dilutions of equine test sera. At least ~~two~~ three replicate wells for each serum dilution are
3. required. Heat-inactivated maintenance medium with a concentration of 2% FCS (HIMM) ~~Serum-free MEM~~ is used
4. throughout as a diluent. Virus stocks of known titre are diluted just before use to contain 100 TCID50 (50% tissue
5. culture infective dose) in 25 µl. Monolayers of ~~E-Derm or~~ RK-13 cells are prepared ~~monodispersed with EDTA/trypsin~~
6. ~~and resuspended at a concentration of 5 × 10~~5~~/ml~~. ~~Note that RK-13 cells can be used with EHV-1 but do not show~~
7. ~~CPE with EHV-4.~~ Antibody positive and negative control equine sera and controls for cell viability, virus infectivity,
8. and test serum cytotoxicity, must be included in each assay. End-point VN titres of antibody are calculated by
9. determining the reciprocal of the highest serum dilution that protects ≥75% ~~100%~~ of the cell monolayer from virus
10. destruction in both of the replicate wells.
11. Serum toxicity may be encountered in samples from horses repeatedly vaccinated with a commercial vaccine
12. prepared from EHV-1 grown up in RK-13 cells. This can give rise to difficulties in interpretation of test reactions at
13. lower serum dilutions. The problem can be overcome using E-Derm or other non-rabbit kidney derived cell line.
14. 2.1.1. Test procedure
15. A suitable test procedure is as follows:
16. i) Prepare semi-confluent monolayers in tissue culture microtitre plates.
17. ii) Inactivate test and control sera for 30 minutes in a water bath at 56°C.
18. iii) Add 40 ~~25~~ µl of HIMM ~~serum-free MEM~~ to all wells of the microtitre assay plates.
19. iv) For test sample titration, pipette ~~25~~ 40 µl of each test serum into ~~duplicate~~ triplicate wells of ~~both~~
20. ~~rows A and B of~~ the plate. The first two rows serve as the dilution of the test serum and the third
21. row serves as the serum toxicity control ~~and the second row as the first dilution of the test~~. Make
22. doubling dilutions of each serum ~~starting with row B and proceeding to the bottom of the plate~~
23. by sequential mixing and transfer of ~~25~~ 40 µl to each subsequent row of wells. ~~Six sera can be~~
24. ~~assayed in each plate.~~ Add 40μl of HIMM to the serum control rows.
25. ~~v)~~ Add 40 ~~25~~ µl of the appropriately diluted EHV-1 ~~or EHV-~~4 virus stock to ~~each~~ all wells
26. (100 TCID50/well) of the test plate except those of ~~row A, which are~~ the serum controls ~~wells~~.
27. Note that the final serum dilutions, after addition of virus, run from a starting dilution of 1/4 ~~to~~
28. ~~1/256~~. A separate control plate should include titration of both a negative and positive (high and
29. low) horse ~~serum~~ sera of known titre, cell control (no virus), and a back titration of virus ~~control~~
30. ~~(no serum), and a virus titration~~ using six wells per log dilution (100 TCID50 to 0.01 TCID50/well)
31. ~~calculate the actual amount of virus used in the test~~
32. vi) Incubate the plates for 1 hour at 37°C in 5% CO2 atmosphere. ~~Add 50 µl of the prepared E-~~
33. ~~Derm or RK-13 cell suspension (5 × 10~~5 ~~cells/ml) in MEM/10% FCS to each well.~~
34. vii) Transfer 50 µl from each well of the test and control plates to the tissue culture microtitre plates.

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1. viii) Incubate the plates for 2 ~~4~~–5 days at 37°C in an atmosphere of 5% CO2 in air.
2. ix) Examine the plates microscopically for CPE and record the results on a worksheet. Confirm the
3. validity of the test by establishing that the working dilution of stock virus is at 100 TCID50/well,
4. that the (high and low) positive control sera are within one well of their pre-determined titre and
5. that the negative control serum is negative at a 1/4 dilution. This takes approximately 72 hours.
6. If at this stage the antigen is too weak the virus concentration may be increased by extending
7. the incubation period up to 5 days. If the antigen is too strong the test must be repeated.
8. Wells are scored as positive for neutralisation of virus if ≥ 75% of the cell monolayer remains
9. intact. The highest dilution of serum resulting in ≥ 75% neutralisation of virus (<25% CPE) in
10. replicate wells is the end-point titre for that serum. Examine the plates microscopically for CPE
11. and record the results on a worksheet.
12. x) Alternatively, the cell monolayers can be scored for CPE after fixing and staining as follows:
13. after removal of the culture fluid, immerse the plates for 15 minutes in a solution containing 2
14. mg/ml crystal violet, 10% formalin, 45% methanol, and 45% water. Then, rinse the plates
15. vigorously under a stream of running tap water. Wells containing intact cell monolayers stain
16. blue, while monolayers destroyed by virus do not stain. ~~Verify that the cell control, positive~~
17. ~~serum control, and serum cytotoxicity control wells stain blue, that the virus control and negative~~
18. ~~serum control wells are not stained, and that the actual amount of virus added to each well is~~
19. ~~between 10~~1.5 ~~and 10~~2.5 ~~TCID50. Wells are scored as positive for neutralisation of virus if 100%~~
20. ~~of the cell monolayer remains intact. The highest dilution of serum resulting in complete~~
21. ~~neutralisation of virus (no CPE) in both duplicate wells is the end-point titre for that serum.~~
22. xi) Calculate the neutralisation titre for each test serum, and compare acute and convalescent
23. phase serum titres from each animal for a four-fold or greater increase.

##### 2.2. Complement fixation test

1. The CF test can be used for the detection and quantification of antibodies against EHV-1. The test determines whether
2. an antigen and an antibody are capable of forming a complex. The presence of an immune complex is revealed by
3. the detector system, which consists of guinea-pig complement and sensitised sheep red blood cells (SRBCs) coated
4. with rabbit haemolytic serum (haemolysin). In the absence of antibodies against equine herpesvirus, no
5. antibody/antigen complex is formed, the complement remains free in the solution and the sensitised SRBCs become
6. lysed. In the presence of antibodies against equine herpesvirus, an antibody/antigen complex is formed, the
7. complement becomes fixed and is therefore unable to lyse the SRBCs. They subsequently form a button at the bottom
8. of the test well.
9. Guinea-pig complement, rabbit haemolytic serum, complement fixation diluent (CFD) and bovine serum albumin
10. (BSA) can be obtained commercially. The dilution of guinea-pig complement that has activity at 3 HD (haemolytic
11. dose) in the presence of sensitised SRBCs should be optimised. The recommended dilution of rabbit haemolytic
12. serum (or the working dilution) is sometimes provided by the supplier. However, the optimal dilution of haemolysin
13. should be determined with the in use reagents (complement etc.) so that the test can be performed reproducibly. The
14. optimum concentration of antigen to be used in the test should be determined using an antigen versus antibody
15. chequerboard technique and by testing a panel of known positive sera.
16. The test is performed in U bottomed microtitre plates. Paired sera should be assayed on the same plate. An antibody
17. positive serum should be included as a control on each plate. All sera are tested on a second plate containing all
18. components except virus to check for anti-complementary activity. A back titration of the working dilution (3 HD) of
19. complement to 2 HD, 1 HD, 0.5 HD is set up in duplicate wells on the complement control plate (eight wells in total).
20. An SRBC control is set up in eight wells.
21. 2.2.1. Preparation of samples
22. i) Samples and controls are prepared by adding 4 volumes (600 µl) of CFD to 1 volume (150 µl)
23. of test sera to give a 1/5 dilution.
24. ii) Diluted serum is inactivated for 30 minutes at 60°C to destroy the naturally occurring
25. complement.
26. 2.2.2. Test procedure
27. i) Prepare the test plate and anti-complementary plate by adding 25 µl 0.05% BSA/CFD to all
28. wells except the first column (H).
29. ii) Add 50 µl of 0.05% BSA/CFD to the eight wells of the complement control (back titration).
30. iii) Add 75 µl of 0.05% BSA/CFD to eight wells of cell control.
31. iv) Add 50 µl of the diluted inactivated test serum and controls to the first well of each row on both
32. the test and anti-complementary plates. Serial doubling dilutions are then made by transferring
33. 25 µl across the plate and discarding the final 25 ml.
34. v) Place the microtitre plates on ice for addition of antigen and complement.
35. vi) Add 25 µl of antigen (diluted to working strength in 0.05% BSA/CFD) to the test plates.
36. vii) Add 25 µl of 0.05% BSA/CFD to all wells of the anti-complementary plate to compensate for
37. lack of antigen.
38. viii) Add 25 µl of guinea-pig complement diluted in 0.05% BSA/CFD to 3 HD to all wells except the
39. complement control and SRBC control.
40. ix) Back titrate the working dilution of 3 HD complement to 2 HD, 1 HD and 0.5 HD in 200 µl
41. volumes. Add 25 µl of each dilution to the appropriate wells.
42. x) Incubate all plates at 4°C overnight.
43. 2.2.3. Preparation and addition of sheep blood
44. i) SRBCs collected into Alsever’s solution are washed twice in 0.05% BSA/PBS solution.
45. ii) Gently resuspend the SRBCs in 5–10 ml 0.05% BSA/CFD solution. Dilute to 2% SRBCS (v/v
46. packed cells) in BSA/CFD solution.
47. iii) Mix the 2% SRBCs with an equal volume of BSA/CFD solution containing haemolysin at its
48. optimal sensitising concentration to give a 1% SRBC solution. Prepare an appropriate volume
49. of this solution by allowing 3 ml per microtitre plate.
50. iv) Incubate at 37°C for 10 minutes. Store the 1% sensitised SRBCs overnight at 4°C.
51. v) The following day, incubate the 1% sensitised SRBCs at 37°C for 30 minutes. During the final
52. 20 minutes of this incubation, transfer the test plates from 4°C to 37°C.
53. vi) At the end of the 30-minute incubation, add 25 ml of 1% sensitised SRBCs to all plates. Mix on
54. a plate shaker for 30 seconds.
55. vii) Incubate the plates at 37°C for 30 minutes. Shake the plates after 15 minutes and at the end of
56. this incubation (a total of three times).
57. viii) Incubate the plates at 4°C for 2 hours to allow the cells to settle.
58. ix) Read and record the test results after 2 hours.
59. 2.2.4. Reading results
60. i) Confirm the validity of the test by establishing that the working dilution of complement is at 3 HD:
61. 100% lysis at 3 HD and 2 HD, and 50% lysis at 1 HD. Distinct buttons should be visible in the
62. eight wells of the SRBC control.
63. ii) There must be 100% lysis observed at the 1/5 dilution for the negative control (<5). The antibody
64. titre of the positive control serum must read within one well of its predetermined titre.
65. iii) Confirm that there are no buttons visible on the anti-complementary plates. Buttoning indicates
66. either the presence of residual native complement in the sample or that there is a non-specific
67. complement fixing effect occurring. Sera that show anti-complementary activity should be
68. retested and treated as described below.
69. iv) In the test wells, buttoning indicates the presence of antibodies in the serum. The antibody titre
70. is the dilution at which there is 50% buttoning and 50% lysis observed.
71. 2.2.5. Treatment of samples showing anti-complementary activity
72. i) Add 50 µl of guinea-pig complement to 150 µl of the serum showing anti-complementary
73. activity.
74. ii) Incubate the sample at 37°C for 30 minutes.
75. iii) Add 550 µl of CFD (1:5 dilution).
76. iv) Heat inactivate at 60°C for 30 minutes.

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**1. Background**

## C. REQUIREMENTS FOR VACCINES

1. Both live attenuated and inactivated vaccines are available for use in horses as licensed, commercially prepared products
2. for use in reducing the impact of disease in horses caused by EHV-1/4 infection. The products contain different
3. permutations of EHV-1 and EHV-4 and some also include equine influenza virus.
4. Clinical experience has demonstrated that vaccination can be useful for reducing clinical signs of respiratory disease and
5. incidence of abortion, however none of the vaccines protects against neurological disease. Multiple doses repeated
6. annually, of each of the currently marketed ER vaccines are recommended by their respective manufacturers. Vaccination
7. schedules vary with a particular vaccine.
8. The indications stated on the product label for use of several available vaccines for ER are either as a preventative of
9. herpesvirus-associated respiratory disease, or as an aid in the prevention of abortion, or both. A minority of ~~Only four~~
10. vaccine products have met the regulatory requirements for claiming efficacy in providing protection from herpesvirus
11. abortion as a result of successful vaccination and challenge experiments in pregnant mares. None of the vaccine products
12. have been demonstrated to prevent the occurrence of neurological disease sometimes associated with EHV-1 infection.
13. Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*.
14. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national
15. and regional requirements.

#### 2. Outline of production and minimum requirements for vaccines

##### 2.1. Characteristics of the seed

1. The master seed virus (MSV) for ER vaccines must be prepared from strains of EHV-1 and/or EHV-4 that have been
2. positively and unequivocally identified ~~by both serological and genetic tests~~. Seed virus must be propagated in a cell
3. line approved for equine vaccine production by the appropriate regulatory agency. A complete record of original
4. source (including isolate number, location, year of isolation), passage history, medium used for propagation, etc.,
5. shall be kept for the master seed preparations of both the virus(es) and cell stock(s) intended for use in vaccine
6. production.
7. 2.1.1. Biological characteristics of the master seed
8. Permanently stored stocks of both MSV and master cell stock (MCS) used for vaccine production
9. must be demonstrated to be pure, safe and, in the case of MSV, also immunogenic.
10. Generally, the fifth passage from the MSV and the twentieth passage from the MCS are the highest
11. allowed for vaccine production. Results of all quality control tests on master seeds must be recorded
12. and made a part of the licensee's permanent records.
13. 2.1.2. Quality criteria
14. Tests for master seed purity include prescribed procedures that demonstrate the virus and cell seed
15. stocks to be free from bacteria, fungi, mycoplasmas, and extraneous viruses. Special tests must be
16. performed to confirm the absence of equine arteritis virus, equine infectious anaemia virus, equine
17. influenza virus, equine herpesvirus-2, -3, and -5, equine rhinitis A and B viruses, the alphaviruses of
18. equine encephalomyelitis, bovine viral diarrhoea virus (BVDV – common contaminant of bovine
19. serum), and porcine parvovirus (PPV – potential contaminant of porcine trypsin). The purity check
20. should also include the exclusion of the presence of EHV-1 from EHV-4 MSV and *vice versa*.
21. 2.1.3. Validation as a vaccine strain
22. Tests for immunogenicity of the EHV-1/4 MSV stocks should be performed in horses on an
23. experimental test vaccine prepared from the highest passage level of the MSV allowed for use in
24. vaccine production. The test for MSV immunogenicity consists of vaccination of horses with low
25. antibody titres to EHV-1/4, with doses of the test vaccine that will be recommended on the final
26. product label. Second serum samples should be obtained and tested for significant increases in
27. neutralising antibody titre against the virus, 21 days after the final dose.
28. Samples of each lot of MSV to be used for preparation of live attenuated ER vaccines must be tested
29. for safety in horses determined to be susceptible to the virulent wild-type virus, including pregnant
30. mares in the last 4 months of gestation. Vaccine safety must be demonstrated in a ‘safety field trial’
31. in horses of various ages from three different geographical areas. The safety trial should be
32. conducted by independent veterinarians using a prelicensing batch of vaccine. EHV-1 vaccines
33. making a claim for efficacy in controlling abortion must be tested for safety in a significant number of
34. late gestation pregnant mares, using the vaccination schedule that will be recommended by the
35. manufacturer for the final vaccine product.

##### 2.2. Method of manufacture

1. 2.2.1. Procedure
2. A detailed protocol of the methods of manufacture to be followed in the preparation of vaccines for
3. ER must be compiled, approved, and filed as an Outline of Production with the appropriate licensing
4. agency. Specifics of the methods of manufacture for ER vaccines will differ with the type (live or
5. inactivated) and composition (EHV-1 only, EHV-1 and EHV-4, EHV-4 and equine influenza viruses,
6. etc.) of each individual product, and also with the manufacturer.
7. 2.2.2. Requirements for ingredients
8. Cells, virus, culture medium, and medium supplements of animal origin that are used for the
9. preparation of production lots of vaccine must be derived from bulk stocks that have passed the
10. prescribed tests for bacterial, fungal, and mycoplasma sterility; nontumorgenicity; and absence of
11. extraneous viral agents.
12. 2.2.3. Final product batch tests
13. i) Sterility
14. Samples taken from each batch of completed vaccine are tested for bacteria, fungi, and mycoplasma
15. contamination. Procedures to establish that the vaccine is free from extraneous viruses are also
16. required; such tests should include inoculation of cell cultures that allow detection of the common
17. equine viruses, as well as techniques for the detection of BVDV and PPV in ingredients of animal
18. origin used in the production of the batch of vaccine.
19. ii) Identity
20. Identity tests shall demonstrate that no other vaccine strain is present when several strains are
21. propagated in a laboratory used in the production of multivalent vaccines.
22. iii) Safety
23. Safety tests shall consist of detecting any abnormal local or systemic adverse reactions to the vaccine
24. in the host species by all vaccination route(s). Tests to assure safety of each production batch of ER
25. vaccine must demonstrate complete inactivation of virus (for inactivated vaccines) as well as a level
26. of residual virus-killing agent that does not exceed the maximal allowable limit (e.g. 0.2% for
27. formaldehyde).
28. iv) Batch potency
29. Batch potency is examined on the final formulated product~~. Batch control of antigenic potency for~~
30. ~~EHV-1 vaccines only may be tested by measuring the ability of dilutions of the vaccine to protect~~
31. ~~hamsters from challenge with a lethal dose of hamster-adapted EHV-1 virus.~~ Although potency
32. testing on production batches of ER vaccine may ~~also~~ be performed by vaccination of susceptible
33. horses followed by assay for seroconversion, ~~the recent availability of virus type-specific MAbs has~~
34. ~~permitted development of less costly and more~~ rapid *in*-*vitro* immunoassays exist for antigenic
35. potency. The basis for such *in-vitro* assays for ER vaccine potency is the determination, by use of
36. ~~the~~ specific MAb, of the presence of at least the minimal amount of viral antigen within each batch of
37. vaccine that correlates with the required level of protection (or seroconversion rate) in a standard
38. animal test for potency.

##### 2.3. Requirements for authorisation/registration/licencing

1. 2.3.1. Manufacturing process
2. For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality
3. control testing (see Sections C.2.1 and C.2.2) should be submitted to the authorities. This information
4. shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the
5. typical industrial batch volume.
6. 2.3.2 Safety requirements
7. Vaccine safety should be evaluated in vaccinated animals using different assays (see Section
8. 2.2.3.iii).
9. 2.3.3 Efficacy requirements
10. Vaccine efficacy (protection) is estimated in vaccinated animals directly by evaluating their resistance
11. to live pathogen challenge.
12. 2.3.4 Duration of immunity
13. As part of the licensing or marketing authorisation procedure, the manufacturer may be required to
14. demonstrate the duration of immunity (DOI) of a given vaccine by either challenge or alternative test
15. at the end of the claimed period of protection.
16. Tests to establish the duration of immunity to EHV-1~~/4~~ or EHV1/4 achieved by immunisation with
17. each batch of vaccine are not required. The results of many reported observations indicate that
18. immunity induced by vaccination-against EHV-1 or EHV induced immunity to EHV-1/4 is not more
19. than a few months in duration; these observations are reflected in the frequency of revaccination
20. recommended on ER vaccine product labels.
21. 2.3.5 Stability
22. As part of the licensing or marketing authorisation procedure, the manufacturer will be required to
23. demonstrate the stability of all the vaccine’s properties at the end of the claimed shelf-life period.
24. Storage temperature shall be indicated and warnings should be given if product is damaged by
25. freezing or ambient temperature.
26. At least three production batches of vaccine should be tested for shelf life before reaching a
27. conclusion on the vaccine’s stability. When stored at 4°C, inactivated vaccine products generally
28. maintain their original antigenic potency for at least 1 year. Lyophilised preparations of the live virus
29. vaccine are also stable during storage for 1 year at 4°C. Following reconstitution, live virus vaccine
30. is unstable and cannot be stored without loss of potency.
31. **Note:** current vaccines are authorised for prevention of respiratory disease or as an aid in the prevention of abortion.
32. Unless the vaccine’s ability to prevent neurological disease is under investigation, the virus used in the challenge
33. experiments should not be a strain with a history of inducing neurological disease.

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1. **NB:** There are WOAH Reference Laboratories for equine rhinopneumonitis (please consult the WOAH Web site:
2. <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).
3. Please contact the WOAH Reference Laboratories for any further information on
4. diagnostic tests, reagents and vaccines for equine rhinopneumonitis
5. and to submit strains for further characterisation.
6. **NB:** First adopted in 1990. Most recent updates adopted in 2017.