Section 3.6

**Equidae**

CHAPTER 3.6.1

# AFRICAN HORSE SICKNESS (INFECTION WITH AFRICAN HORSE SICKNESS VIRUS)

## SUMMARY

***Description of the disease:*** *African horse sickness (AHS) is an infectious but noncontagious viral disease especially affecting horses, although all species of the family equidae are susceptible to infection. It is caused by an orbivirus of the family Sedoreoviridae. Disease, when present, varies from mild fever, depending on the levels of immunity and the equid species involved, to severe breakdown of the respiratory and circulatory systems resulting in death.*

*All serotypes of AHS occur in eastern and southern Africa. AHS serotypes 2, 4 and 9 have been found in North and West Africa, from where they occasionally spread into countries surrounding the Mediterranean. Examples of outbreaks that have occurred outside Africa are: in the Middle East* (serotype 9, *1959–1963*), in Asia (Thailand, serotype 1, 2020), *in Spain* (*serotype 9, 1966, serotype 4, 1987–1990*), *and in Portugal* (*serotype 4, 1989*).

*Laboratory diagnosis of AHS is essential. Although the clinical signs and lesions are characteristic of the severe forms of the disease, such signs can still be confused with those of other equine diseases.*

*As a viral disease, the laboratory diagnosis of AHS can be based on the identification of infectious virus, virus nucleic acid, viral antigens or specific antibodies. A wide variety of laboratory tests have been adapted for the detection of both AHS virus (AHSV) and specific antibodies.*

***Detection and identification of the agent:*** *a rapid and highly sensitive diagnosis is usually achieved by nucleic acid viral detection using reverse-transcription polymerase chain reaction (PCR). Nevertheless, it is particularly important to perform virus isolation and serotyping whenever AHS outbreaks occur outside the enzootic regions in order to choose a homologous serotype for the vaccine.*

*AHSV can be isolated from blood collected during the early febrile stage. For virus isolation, the other tissues of choice for diagnosis are spleen, lung, and lymph nodes, collected at necropsy. Sample preparations can be inoculated in cell cultures, such as baby hamster kidney-21 (BHK-21), monkey stable (MS), African green monkey kidney (Vero) or insect cells (KC) or intravenously into embryonated eggs. Virus isolates can be serotyped by a type-specific serological test such as virus neutralisation (VN), by type-specific reverse-transcription PCR or by sequencing. Molecular typing assays can also be carried out on clinical samples.*

*Other methods such as enzyme-linked immunosorbent assays (ELISAs) for the rapid detection of AHSV antigen in spleen tissues and supernatant from infected cells have been developed although they are currently out of use due to their limited sensitivity.*

***Serological tests:*** *Horses that survive natural infection develop antibodies against the infecting serotype of AHSV within 8–12 days post-infection. This may be demonstrated by several*

*serological methods, such as complement fixation test, ELISA, immunoblotting and VN. The latter test can be used for serotyping.*

***Requirements for vaccines:*** *Attenuated (monovalent and polyvalent) live vaccines for use in horses, mules and donkeys, are currently commercially available. Inactivated vaccines were used in the past and recombinant approaches have been evaluated experimentally.*

## A. INTRODUCTION

### 1. Description and impact of the disease

African horse sickness (AHS) (Peste equina africana, Peste equine) is an infectious, non-contagious arthropod-borne disease especially of horses, although all equids are susceptible to infection, which is caused by a double-stranded RNA orbivirus belonging to the family Sedoreoviridae. At least two field vectors are involved in the transmission of the virus: *Culicoides imicola* and *C. bolitinos*.

AHS is enzootic in sub-Saharan Africa, although occasional outbreaks have occurred in northern Africa (1965, 1989–1990, 2007–2010), the Middle East (1959–1961), Asia (Thailand 2020), and in Europe (Spain: 1966, 1987–1990, and Portugal: 1989) (Lu *et al*, 2020).

There are four classical clinical forms of AHS: pulmonary, cardiac, mixed, and horse sickness fever. The peracute, pulmonary form occurs in fully susceptible animals and has a short course, often only a few hours, and a high mortality rate. The animal exhibits respiratory distress, an extended head and neck, and profuse sweating. Terminally, froth exudes from the nostrils. The cardiac, oedematous form has a more subacute course with mortality reaching 50%. The head and neck may show severe swelling that can extend down to the chest. Swelling of the supraorbital fossae is characteristic and may include conjunctival swelling with petechiae. Paralysis of the oesophagus may result in aspiration pneumonia and sublingual haemorrhages are always a poor prognostic sign. The mixed, acute form is most commonly seen and has features of both the cardiac and pulmonary forms. Mortality can reach 70%. Horse sickness fever is an often overlooked, mild form of the disease and is seen in partially immune horses and more resistant equids such as donkeys, as well as in zebra post-experimental infection (Coetzer & Guthrie, 2005).

Clinical cases have also been described in dogs, with acute respiratory distress syndrome or sudden death. The mortality in dogs is high, and they may play a role in spread of the disease. Historically, infection was attributable to the consumption of infected horse meat, however more recent evidence includes the suspicion of vector-transmission (O'Dell *et al.,* 2018).

The disease has both a seasonal (late summer/autumn) and a cyclical incidence with major epizootics in southern Africa during warm-phase events, such as occurrences of El Niño. Mortality due to AHS is related to the equid species affected, any existing prior immunity (e.g. due to vaccination), and to the strain or serotype of the virus. Among equidae, horses are the most susceptible to AHS with a mortality rate of 50–95%, followed by mules with mortality around 50%. In enzootic regions of Africa, donkeys are very resistant to AHS and experience only subclinical infections. In European and Asian countries, however, donkeys are moderately susceptible and have a mortality rate of 10%. Zebras are also markedly resistant with no clinical signs, except for fever, although this has only been observed after experimental inoculation, and they may also have an extended viraemia (up to 40 days).

Attenuated (monovalent and polyvalent) live vaccines for use in horses, mules and donkeys, are currently commercially available.

### 2. Nature and classification of the pathogen

AHS is caused by African horse sickness virus (AHSV) a member of the family Sedoreoviridae, genus Orbivirus, and species *Orbivirus alphaequi*. The genus *Orbivirus* also includes the viruses that cause bluetongue and epizootic haemorrhagic disease, which have similar morphological and biochemical properties with distinctive pathological and antigenic properties as well as host ranges. Nine antigenically distinct serotypes of AHSV have been identified by virus neutralisation; some cross-reaction has been observed between 1 and 2, 3 and 7, 5 and 8, and 6 and 9, but no cross-reactions with other known orbiviruses occur. The virus can be inactivated at 72°C for 120 minutes (confirmed by three blind passages in the Vero cell line).

The virion is an unenveloped particle of a size around 70 nm. The genome of AHSV is composed of ten double-stranded RNA segments, encoding seven structural proteins (VP1-7), and four nonstructural proteins (NS1, NS2, NS3, NS3A). Proteins VP2 and VP5 form the outer capsid of the virion, and proteins VP3 and VP7 are the major inner capsid proteins. Proteins VP1, VP4 and VP6 constitute minor inner capsid proteins (Roy *et al*., 1994). The NS3 proteins are the second most variable AHSV proteins, the most variable being the major outer capsid protein, VP2. This protein, VP2, is the determinant of AHSV serotypes and, together with VP5, the target for virus neutralisation activity.

### 3. Zoonotic potential and biosafety and biosecurity requirements

There is no evidence that humans can become infected with any field strain of AHSV, either through contact with naturally or experimentally infected animals or by virus manipulation in laboratories. However, infection by vaccine strains of AHSV has been described in four laboratory workers of a vaccine-packing facility, but it must be stressed that the infections described occurred under particular circumstances with known encephalitogenic strains and there is no evidence to suggest that AHS virus should ordinarily be considered a human pathogen (van der Meyden *et al.*, 1992). Laboratory manipulations should be performed with appropriate containment determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

### 4. Differential diagnosis

A laboratory diagnosis is essential to establish a correct and confirmatory diagnosis. Although some clinical signs and lesions are characteristic, AHS can be confused with other diseases. For example, the supraorbital swelling, which is often present in horses with subacute AHS, is, in combination with an appropriate history, sufficient for a tentative diagnosis. Other signs and lesions are less specific for AHS, and other diseases such as equine encephalosis, equine infectious anaemia, Hendra virus, equine viral arteritis, piroplasmosis and purpura haemorrhagica should be excluded (WOAH, 2010).

## B. DIAGNOSTIC TECHNIQUES

***Table 1. Test methods available for the diagnosis of African horse sickness and their purpose***

| Method | Purpose | | | | | |
|---|---|---|---|---|---|---|
| | **Population freedom from infection[a]** | **Individual animal freedom from infection prior to movement[b] [c]** | **Contribute to eradication policies[d]** | **Confirmation of clinical cases[e]** | **Prevalence of infection – surveillance[f]** | **Immune status in individual animals or populations post-vaccination[g]** |
| Detection and identification of the agent[h] | | | | | | |
| Real-time RT-PCR | ☑☐☐ | ☑☑☑ | ☑☑☑ | ☑☑☑ | ☑☑☐ | ☐☐☐ |
| Agarose gel-based RT-PCR | ☑☐☐ | ☑☑☐ | ☑☑☐ | ☑☑☐ | ☑☐☐ | ☐☐☐ |
| Virus isolation | ☐☐☐ | ☑☐☐ | ☐☐☐ | ☑☑☑ | ☐☐☐ | ☐☐☐ |
| Detection of immune response | | | | | | |
| ELISA (serogroup specific based on VP7) | ☑☑☑ | ☑☑☑ | ☑☑☐ | ☑☐☐ | ☑☑☑ | ☑☑☐ |
| CFT | ☑☐☐ | ☑☐☐ | ☑☐☐ | ☑☐☐ | ☑☐☐ | ☑☐☐ |
| VN | ☑☐☐ | ☑☐☐ | ☐☐☐ | ☑☐☐ | ☑☐☐ | ☑☑☑ |

Key: ☑☑☑ = recommended for this purpose; ☑☑□ = recommended but has limitations;
☑□□ = suitable in very limited circumstances; □□□ = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay;
VN = virus neutralisation; 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) For serological tests, paired samples may be required as recommended in the *Terrestrial Code* Chapter 12.1. 'Infection with African horse sickness virus', Article 12.1.7 'Recommendations for importation from AHS infected countries or zones'

(d) See Appendix 3 of this chapter for justification table for the scores given to the tests for this purpose.

(e) See Appendix 4 of this chapter for justification table for the scores given to the tests for this purpose.

(f) See Appendix 5 of this chapter for justification table for the scores given to the tests for this purpose.

(g) See Appendix 6 of this chapter for justification table for the scores given to the tests for this purpose.

(h) A combination of agent identification methods applied on the same clinical sample is recommended.

If possible, more than one test should be performed to diagnose an outbreak of AHS, especially the index case. The initial test can be a polymerase chain reaction (PCR), followed by virus isolation in tissue culture. Serological techniques for serogroup specific detection enzyme-linked immunosorbent assay (ELISA) can also be used as a complementary method to detect infection by the detection of seroconversion, for which paired samples should be analysed. Virus neutralisation (VN) for serotype identification, type-specific reverse-transcription (RT)-PCR or sequencing should be performed as early in the outbreak as possible so that the serotype can be identified and the correct vaccine selected.

At present, international standards, both viral and antibody panels, for the identification of AHSV and specific antibodies, are available at the European Union (EU) Reference Laboratory (RL) for AHS. Inactivated virus of serotypes 1–9 reference strains can be obtained from the WOAH Reference Laboratory in Spain to set up the RT-PCR detection method and establish its analytical sensitivity in comparison with real-time RT-PCR procedure (Agüero *et al.,* 2008). These materials have been distributed worldwide.

Since 2007, proficiency tests have been organised annually by the EU-RL for AHSV antibody determination and viral genome detection. Laboratories involved in AHS official control around the world have participated. A commercial blocking ELISA for AHSV antibodies detection has been widely used by participants and the results have demonstrated a good performance. Regarding viral genome detection, real-time RT-PCR methods have been widely used and similar results have been obtained with different RT-PCR assays.

A key aspect of the diagnosis is the selection of samples and their correct storing and safe transporting. Orbiviruses remain viable at 4°C in ethylene diamine tetra-acetic acid (EDTA) blood samples for weeks or at −80°C for long-term storage. Organ samples should be frozen at −80°C for 48–72 hours. Serum samples for antibody detection should be kept at −20°C if storage is required for >1 week. Both the International Air Transport Association (IATA) and the European Agreement concerning the International Carriage of Dangerous Goods by Road (ADR) categorise clinical samples and culture containing AHSV as B UN 3373 (see Chapter 1.1.3 *Transport of biological materials*).

## 1. Identification of the agent

### 1.1. Virus isolation

Unclotted whole blood collected during the early febrile stage of the disease from sick animals, as well as small pieces (2–4 g) of spleen, lung and lymph nodes from animals that have died, are the samples of choice for diagnosis. Samples should be kept at 4°C during transportation and short-term storage prior to processing.

#### 1.1.1. Cell culture

Successful direct isolation of AHSV has been performed on baby hamster kidney (BHK-21), monkey stable (MS) and African green monkey kidney (Vero) mammalian cell lines and on *Culicoides* and mosquito insect cell lines. Blood samples collected in an appropriate anticoagulant can be used diluted as the inoculum. After 15–60 minutes of

adsorption at ambient temperature or at 37°C, the cell cultures are washed and maintenance medium is added. Alternatively, and more commonly, the blood is washed, lysed and diluted 1/10. This procedure removes unwanted antibody, which could neutralise free virus, and promotes release of virus associated with the red blood cell membranes. When tissue samples, such as spleen, lung, etc., are used, a 10% tissue suspension is prepared in phosphate buffered saline (PBS) or cell culture medium, containing antibiotics.
A cytopathic effect (CPE) may appear between 2 and 10 days post-infection with mammalian cells. Three blind passages should be performed before considering the samples to be negative. No CPE is observed in insect cells but the presence of the virus can be detected in the supernatant after 5–7 days by real-time RT-PCR. Supernatant from infected insect cells can then be passed onto mammalian cells, which will show CPE after one or two passages. Positive CPE must be confirmed by real time RT-PCR or VNT. Real-time RT-PCR results should be carefully interpreted as positive results do not necessarily indicate the presence of infectious virus.

## 1.2. Nucleic acid methods

### 1.2.1. Reverse-transcription polymerase chain reaction

RT-PCR is a highly sensitive technique that provides a rapid identification of AHS viral nucleic acid in blood and other tissues of infected animals. The RT-PCR procedure will detect virus-specific nucleic acid after the virus is no longer viable and capable of establishing a new infection in either insects or mammalian cells.

Several agarose gel-based RT-PCR assays for the specific detection of AHSV RNA have been described targeted at viral segments 3, 7 or 8 (Aradaib, 2009; Bremer *&* Viljoen, 1998; Zientara *et al*., 1994). The most widely used method employs primers corresponding to the 5' end (nucleotides 1–21) and 3' end (nucleotides 1160–1179) of RNA segment 7 (coding for VP7) amplifying the complete viral segment (Zientara *et al.,* 1994).

Real-time RT-PCR methods for the highly sensitive and specific detection of AHSV RNA have been developed based on the use of a pair of primers and a labelled probe from conserved sequences of viral segments 3, 5 or 7 (Agüero *et al*., 2008; Bachanek-Bankowska *et al.,* 2014). A duplex real-time RT-PCR has also been described that targets segments 7 and 8 of the genome (coding for VP7 and NS2 respectively) (Quan *et al*., 2010).

Although both gel-based and real-time RT-PCR procedures can detect reference strains from the nine virus serotypes, real-time RT-PCR provides advantages over agarose gel-based RT-PCR methods, with its faster analysis time, higher sensitivity, and suitability for high-throughput automation. Nevertheless, gel-based RT-PCR methods, particularly those amplifying long RNA fragments (Zientara *et al*., 1994), can be very useful in the further genetic characterisation of the virus by sequencing of the amplicons. In addition, it may be beneficial in laboratories without the capacity to perform real-time RT-PCR.

In 2015 the WOAH Reference Laboratories for AHS carried out an international ring trial to gather information on the performance of the different methods used in the main AHSV diagnostic laboratories. Ten different RT-PCR protocols were evaluated. Although in this trial some methods could only be tested in one or two laboratories, they produced very good results and therefore are suitable for further evaluation and validation. The study identified that the real-time RT-PCR methods of Agüero *et al.* (2008) and Guthrie *et al.* (2013) correctly detected all the representative strains included in the international ring trial with a high sensitivity in the analysis of field samples. These methods are validated for certification of individual animals prior to movement and are described below.

Details of AHSV gel-based RT-PCR and real-time RT-PCR methods are given below.

To assure a good reaction it is necessary to extract from the sample an AHSV RNA of high quality. The extraction of nucleic acids from clinical samples can be performed by a variety of in-house and commercially available methods.

Denaturation of extracted RNA has to be performed prior to the RT-PCR procedure as the AHSV genome consists of double-stranded RNA.

### 1.2.2. Agarose gel-based RT-PCR procedure (Zientara *et al.,* 1994)

| Pathogen/ target gene | Primer (5'–3') | Concentration | Cycling parameters[a] |
|---|---|---|---|
| Method 1: Zientara *et al.,* 1994; amplicon size: 1179 bp | | | |
| AHSV segment 7 (VP7) | Fwd: GTT-AAA-ATT-CGG-TTA-GGA-TG<br>Rev: GTA-AGT-GTA-TTC-GGT-ATT-GA | 250 nM (each primer) | 37°C/1 hour or 55°C/45min; 95°C/5–10min;<br>40 cycles of: 94–95°C/1 min, 55°C/1–1.5 min,<br>70–72°C/2–2.5 minutes;<br>final extension step: 70–72°C/7–8min |

[a]A denaturation step prior to cycling has not been included

### 1.2.3. Real-time RT-PCR procedure

These group-specific real-time RT-PCRs have been employed with very good results by the participating national reference laboratories of the EU Member States in annual proficiency tests for the period 2009–2024. Moreover, in an international ring trial organised in 2015 under the auspices of the WOAH Reference Laboratory network, both of them were found to be among other top-ranking protocols.

It is highly recommended to include an internal control in the PCR to detect inhibitors in the sample that may produce a false negative result. The method, which has been optimised for both VP7 and VP7 plus β-actin, is able to detect all known AHSV types and strains currently circulating.

| Pathogen/ target gene | Primer/probe (5'–3') | Concentration | Cycling parameters[a] |
|---|---|---|---|
| Method 1: Agüero *et al.,* 2008 | | | |
| AHSV segment 7 (VP7) | Fwd: CCA-GTA-GGC-CAG-ATC-AAC-AG<br>Rev: CTA-ATG-AAA-GCG-GTG-ACC-GT<br>Probe: FAM–GCT-AGC-AGC-CTA-CCA-CTA–MGB | 1000 nM (each primer)<br>250 nM (probe) | 48°C/25 min; 96°C/10 min; 40 cycles:<br>95°C/15 sec, 55°C/35 sec, 72°C/30 sec |
| Method 2: Guthrie *et al.,* 2013 | | | |
| AHSV segment 7 (VP7) | Fwd: AGA-GCT-CTT-GTG-CTA-GCA-GCC-T<br>Rev: GAA-CCG-ACG-CGA-CAC-TAA-TGA<br>Probe: FAM–TGC-ACG-GTC-ACC-GCT–MGB | 200 nM (each primer)<br>120 nM (probe) | 48°C/10 min; 95°C/10 min; 40 cycles:<br>95°C/15 sec, 60°C/45 sec |

i) Interpretation of the results

**Note:** the positive/inconclusive/negative cut-off values shown should be validated or adjusted in individual laboratories according to the reagents and equipment in use.

The assay is considered not valid if atypical amplification curves are obtained. If this is the case, the assay must be repeated.

The assay is considered positive when a typical amplification curve is obtained and the Ct value (the number of polymerase chain reaction (PCR) cycles required for fluorescent signal to exceed the background) is lower or equal to the defined Ct threshold (35) within 40 PCR cycles (Ct≤35).

The assay is considered inconclusive when a typical amplification curve is obtained and the Ct value is higher than the defined Ct threshold (35) within 40 PCR cycles (Ct≥35).

The assay is considered negative when a horizontal amplification curve is obtained and does not cross the threshold line within 40 PCR cycles and the Ct for the internal control (β-actin) is lower or equal to 32.

### 1.3. AHSV typing

Until recently, the VN test has been the method of choice for typing as well as the 'gold' standard test for identifying AHSV isolated from the field using type-specific antisera (Verwoerd, 1979). This technique takes 5 or more days before results are obtained. The development of type-specific gel-based RT-PCR (Maan *et al.,* 2011; Van Schalkwyk *et al*., 2019), and real-time RT-PCR using hybridisation probes (Koekemoer, 2008) targeting AHSV Seg-2 for identification and differentiation of AHSV genotypes, as well as the more recently described type-specific real-time RT-PCR assays based on the use of labelled DNA probes–MGB probes (Bachanek-Bankowska *et al*., 2014; Villalba *et al*., 2024; Weyer *et al.,* 2015) provides a rapid typing method for AHSV in tissue samples and blood. In comparison to VN tests, these methods can be used to significantly increase the speed and reliability of detection and identification of the nine serotypes of AHSV.

However, the genetic variation that may appear over time in the AHSV genome, specifically in the VP2 coding region, where specific primers/probes for typing assays have to be designed, makes the detection of all genetic variants within each serotype by this type of technique difficult. Therefore, although molecular methods can rapidly type AHSV in many positive field samples, VN should be kept as the gold standard for serotyping AHSV isolates.

## 2. Serological tests

Indirect and competitive blocking ELISAs using either soluble AHSV antigen or a recombinant protein VP7 (Laviada *et al*., 1992; Maree & Paweska, 2005) have proved to be good methods for the detection of anti-AHSV group-reactive antibodies, especially for large-scale investigations. The competitive blocking ELISA can also be used for testing wildlife as species-specific anti-globulin is not required with this method. An immunoblotting test has also been adapted for anti-AHS antibody determination (Laviada *et al*., 1992), which is especially suitable for small numbers of sera. The complement fixation (CF) test has been widely used, but some sera are anti-complementary, particularly donkey and zebra sera.

### 2.1. Blocking enzyme-linked immunosorbent assay

The competitive blocking ELISA technique detects specific antibodies against AHSV, present in any equine species. VP7 is the main antigenic protein within the molecular structure of AHSV and it is conserved across the nine AHSV serotypes. An MAb directed against VP7 is used in this test, allowing high sensitivity and specificity. Moreover, other species of the family Equidae (e.g. donkeys, zebra, etc.) can be tested thus preventing the problem of specificity experienced occasionally using the indirect ELISAs. VP7 recombinant antigen is non-infectious, which provides a high level of security.

The principle of this test is to block the specific reaction between the recombinant VP7 protein absorbed on an ELISA plate and a conjugated MAb against VP7. AHSV antibodies present in a suspect serum sample will block this reaction. A decrease in the amount of colour is evidence of the presence of AHSV antibodies in the serum sample.

The competitive blocking ELISA is commercially available. The reproducibility of the test was assessed in an international ring trial (Durán-Ferrer *et al.,* 2019).

#### 2.1.1. Test procedure

The test described here is an example of a blocking ELISA.

i) *Solid phase:* coat 96-well ELISA plates with 50–100 ng of recombinant AHSV VP7 diluted in carbonate/bicarbonate buffer, pH 9.6. Incubate overnight at 4°C.

ii) Wash the plates three times with PBS 0.1× containing 0.135 M NaCl and 0.05% (v/v) Tween 20 (washing solution). Gently tap the plates onto absorbent material to remove any residual wash.

iii) *Test samples:* serum samples to be tested, and positive and negative control sera (if not ready to use by kit manufacturer), are diluted 1/5 in diluent containing 0.35 M NaCl, 0.05% Tween 20; and 0.1% Kathon, 100 µl per well. Incubate for 1 hour at 37°C.

iv) Wash the plates five times with PBS 0.1× containing 0.135 M NaCl and 0.05% (v/v) Tween 20 (washing solution). Gently tap the plates onto absorbent material to remove any residual wash.

v) *Conjugate:* dispense 100 µl/well of horseradish peroxidise-conjugated MAb anti-VP7 at optimal dilution in a suitable diluent. The MAb and diluent may be included in commercial kits. Incubate for 30 minutes at 37°C.

vi) Wash the plates as described in step iv.

vii) *Substrate/chromogen:* add 100 µl/well substrate/chromogen solution, e.g. ABTS (2,2'-azino-di-[3-ethyl-benzothiazoline]-6-sulphonic acid) 5 mg/ml diluted 1/10 in 0.1 M phosphate/ citrate buffer, pH 4, containing 0.03% $H_2O_2$, and incubate for 10 minutes at room temperature.

Colour development is stopped by adding 100 µl/well of 2% (w/v) of SDS. Alternative chromogen systems may be used (e.g. tetramethyl benzidine).

viii) Read the plates at 405 nm.

ix) *Validation of the assay:* positive control lower than 0.2 and negative control higher than 1.0.

x) *Interpretation of results:* determine the blocking percentage (BP) of each sample by applying the following formula:

$$BP = \frac{\text{Abs (Control Neg)} - \text{Abs (sample)}}{\text{Abs (Control Neg)} - \text{Abs (Control Pos)}} \times 100$$

Samples showing BP value lower than 45% are considered negative for antibodies to AHSV. Samples showing BP value higher than 50% are considered positive for antibodies to AHSV. Samples with BP value between 45% and 50% are considered doubtful and must be retested. If the result is the same, resample and test 2 weeks later.

### 2.2. Indirect enzyme-linked immunosorbent assay

The recombinant VP7 protein has been used as antigen for AHSV antibody determination with a high degree of sensitivity and specificity (Laviada *et al.,* 1992; Maree & Paweska, 2005). Other advantages of this antigen are its stability and its lack of infectivity. The conjugate used in this method is a horseradish peroxidase anti-horse gamma-globulin reacting with horse, mules and donkeys. The method described by Maree & Paweska (2005) uses protein G as conjugate that also reacts with zebra serum. The indirect ELISA is recently also commercially available.

#### 2.2.1. Test procedure

There are several test procedures described; this is an example of one AHS indirect ELISA procedure.

i) *Solid phase:* Coat 96-well ELISA plates with recombinant AHSV-4 VP7 diluted in carbonate/bicarbonate buffer, pH 9.6. Incubate overnight at 4°C.

ii) Wash the plates five times with distilled water containing 0.01% (v/v) Tween 20 (washing solution). Gently tap the plates on to absorbent material to remove any residual wash.

iii) Block the plates with PBS, pH 7.2 + 5% (w/v) skimmed milk, 200 µl/well, for 1 hour at 37°C.

iv) Remove the blocking solution and gently tap the plates on to absorbent material.

v) *Test samples:* Serum samples to be tested, and positive and negative control sera, are diluted 1/25 in PBS + 5% (w/v) skimmed milk + 0.05% (v/v) Tween 20, 100 µl per well. Incubate for 1 hour at 37°C. For titration, add twofold dilution series from 1/25 (100 µl/well), one serum per plate column, in duplicate columns, and do the same with positive and negative controls. Incubate for 1 hour at 37°C.

vi) Wash the plates as described in step ii.

vii) *Conjugate:* Dispense 100 µl/well of horseradish peroxidase conjugated anti-horse gamma-globulin diluted in PBS + 5% milk + 0.05% Tween 20, pH 7.2. Incubate for 1 hour at 37°C.

viii) Wash the plates as described in step ii.

ix) *Chromogen/Substrate:* Add 200 µl/well of chromogen/substrate solution (10 ml 80.6 mM DMAB [3-(dimethylamino) benzoic acid] + 10 ml 1.56 mM MBTH [3-methyl-2-benzothiazolinone hydrazone] + 5 µl $H_2O_2$). Colour development is stopped by adding 50 µl of 3 N $H_2SO_4$ after approximately 5–10 minutes (before the negative control begins to be coloured). Other chromogens such as ABTS, tetramethyl benzidine or orthophenyldiamine can also be used.

x) Read the plates at 600 nm, 620 nm, or 450nm, depending on the chromogen used.

xi) *Interpretation of results:* Calculate the cut-off value by adding 0.06 to the value of the negative control. (0.06 is the standard deviation derived with a group of 30 negative sera) Test samples giving absorbance values lower than the cut-off are regarded as negative. Test samples giving absorbance values greater than the cut-off + 0.15 are regarded as positive. Test samples giving intermediate absorbance values are doubtful and a second technique must be employed to confirm the result.

## 2.3. Complement fixation

The CF test has been used extensively in the past, but currently its use is decreasing and has been replaced in many laboratories by ELISA as a screening technique. This progressive replacement is because of the higher sensitivity and degree of standardisation of ELISA as well as a significant number of sera with anti-complementary activity. Nevertheless, the CF test is a useful tool in endemic areas for the demonstration and titration of group-specific IgM antibodies against AHSV notably following a recent infection or vaccination.

### 2.3.1. Reagents

i) Veronal buffered saline containing 1% gelatin (VBSG).

ii) Serum samples, free from erythrocytes, must be heat inactivated: horse serum at 56°C, zebra serum at 60°C and donkey serum at 62°C, for 30 minutes.

iii) The antigen is a sucrose/acetone extract of AHSV-infected mouse brain. The control antigen is uninfected mouse brain, extracted in the same way. In the absence of an international standard serum, the antigen should be titrated against a locally prepared positive control serum. In the test, four to eight units are used. The antigen may also be obtained by inoculation of the virus in suitable cell culture (see Section B.1 above).

iv) The complement is a normal guinea-pig serum.

v) The haemolysin is a hyperimmune rabbit serum against sheep red blood cells (SRBCs).

vi) The SRBCs are obtained by aseptic puncture of the jugular vein and preserved in Alsever's solution[1] or sodium citrate.

vii) The haemolytic system (HS) is prepared by diluting the haemolysin to contain two haemolytic doses and using this to sensitise washed SRBCs. The SRBCs are standardised to a 3% concentration.

viii) *Control sera:* A positive control serum is obtained locally and validated. Serum from a healthy antibody-negative horse is used as the negative control serum.

### 2.3.2. Test procedure

i) The reaction is performed in 96-well round-bottom microtitre plates in a final volume of 100 µl/well or in tubes if the macro-technique is used, at 4°C for 18 hours.

ii) All the sera, samples and controls are diluted 1/5 in VBSG and 25 µl of each serum is added in duplicate. A twofold dilution series of each serum is done from 1/5 to 1/160.

iii) Add 25 µl of the antigen diluted according to the previous titration.

iv) Add 25 µl of the complement diluted according to a previous titration.

v) Incubate at 4°C for 18 hours.

vi) 25 µl of HS is added to all wells on the microtitre plate.

vii) The plate is incubated for 30 minutes at 37°C.

viii) Plates are then centrifuged at 200 ***g***, and the wells are scored for the presence of haemolysis. Control of sera, complement, antigen and HS are used

ix) Results are read using 50% haemolysis as the end point. The inverse of the highest dilution of serum specifically fixing complement with the CF antigen is called the titre.

x) A titre of 1/10 or more is positive, under 1/10 is negative.

### 2.4. Virus neutralisation serology

Serotype-specific antibody can be detected using the VN serology test (House *et al.*, 1990). The VN serology may have additional value in epidemiological surveillance and transmission studies, mainly in endemic areas where multiple serotypes are likely to be present.

#### 2.4.1. VN serology test procedure

i) From a starting dilution of 1/5, serial twofold dilutions of the test sera are made in a cell-culture grade flat-bottomed 96-well microtitre plate, using cell culture medium as diluent. For each sample two wells are used at each dilution. Control positive and negative sera should also be included in each batch of tests. An equal volume (e.g. 25 µl) of a stock of AHSV containing 100 $TCID_{50}$ (50% tissue culture infective dose) is added to each well.

ii) Serum/virus mixtures are incubated for 60 minutes at 37°C 5% $CO_2$ and 95% humidity prior to the addition of 0.1 ml of Vero cell suspension (200,000 cells/ml) to each test well.

iii) A back titration of virus stock is prepared for each test using four wells per tenfold dilution, 25 µl per well. Test plates are incubated at 37°C, 5% $CO_2$, 95% humidity for 4–5 days, until the back titration indicates that the stock virus contains 30–300 $TCID_{50}$.

iv) After incubation for 4–5 days, the test is read using an inverted microscope. Wells are scored for the presence or absence of CPE. The presence of CPE in the wells containing the serum sample indicates that the tested serum does not contain specific neutralising antibodies against the virus, therefore producing cell lysis with the consequent destruction of the cell layer.

By contrast, the absence of CPE in the wells containing the serum sample indicates that the tested serum does contain specific neutralising antibodies against the virus, therefore maintaining intact the cell layer.

v) Alternatively, the plates are then fixed and stained in a solution of 0.15% (w/v) crystal violet in 2% (v/v) glutaraldehyde and rinsed or they may be fixed with 70% ethanol and stained with 1% basic fuchsin.

vi) The 50% end-point titre of the serum is calculated by the Spearman–Kärber method and expressed as the negative $\log_{10}$.

## C. REQUIREMENTS FOR VACCINES

## 1. Background

### 1.1. Rationale and intended use of the product

Polyvalent or monovalent live attenuated AHS vaccines, based on the selection in Vero cell culture of genetically stable macroplaques, have been used for the control of AHSV in and out of Africa. Polyvalent vaccines are commercially available.

It must be noted that detection of viral genome in animals vaccinated with live attenuated AHS vaccines has been described up to 16 weeks after vaccination (Weyer *et al.,* 2017).

An inactivated monovalent (serotype 4) AHSV vaccine based on virus purification and inactivation with formalin was produced commercially in the early 1990s (House *et al*., 1992), but is not commercially available at the present time. More recently, inactivated vaccines have been produced and successfully used against all serotypes (Rodríguez *et al.,* 2020). Subunit AHSV vaccines based on serotype 4 outer capsid protein VP2 and VP5 plus inner capsid protein VP7, derived from single and dual recombinant baculovirus expression vectors have been used experimentally in different combinations to immunise horses (Martinez *et al*., 1996). The protective efficacy of VP2 in a subunit vaccine was also evaluated (Scanlen *et al*., 2002). However, these vaccines are not commercially available. Other examples of experimental new generation vaccines described but currently not commercially available, include virus-vectored vaccines (e.g. modified vaccinia Ankara virus: Alberca *et al.,* 2014), plant-derived virus-like particles (O'Kennedy *et al.,* 2024) as well as entry-competent replicative-abortive (ECRA) and disabled infection single animal (DISA) reverse genetics vaccine platforms (Van Rijn *et al.,* 2018).

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

At present only the live attenuated AHS vaccines (polyvalent or monovalent) are commercially available. 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.

## 3. Live attenuated African horse sickness vaccine

### 3.1. Characteristics of the seed

#### 3.1.1. Biological characteristics

The seed virus is prepared by selection in Vero cells of genetically stable large plaques from low passage levels of AHSV. The plaque mutants are then further multiplied by three passages in Vero cells. A large quantity of this antigen is lyophilised and stored at –20°C as seed stock antigen.

#### 3.1.2. Quality criteria

The seed virus must be shown to be free of contaminating viruses, bacteria and mycoplasmas by the appropriate techniques. The serotype identity of the seed virus is confirmed.

### 3.2. Method of manufacture

#### 3.2.1. Procedure

At the onset of a production run, working antigens are produced from the seed stock antigen in either BHK-21 or Vero cell cultures. The working antigens are tested for sterility, purity and identity and should contain at least $1 \times 10^6$ plaque-forming units (PFU)/ml of infectious virus.

#### 3.2.2. Requirements for substrates and media

Roller bottle cultures of Vero or BHK-21 cells are grown using gamma-irradiated bovine serum in the growth medium. Once the cultures are confluent, the medium is poured off and the cells are seeded with the working antigens. After 1 hour, maintenance medium is added to the cultures. Incubation is continued at 37°C for 2–3 days. When the CPE is advanced, both cells and supernatant medium are harvested. The products from the same serotype are pooled and stored at 4°C.

#### 3.2.3. In-process control

The pooled harvests of the individual serotypes are tested for sterility and assayed for infectivity by plaque titration on Vero cell cultures. The minimum acceptable titre is $1 \times 10^6$ PFU/ml.

Finally, two multivalent vaccines are constituted by mixing equal volumes of serotypes 1, 3, 4 and 2, 6, 7, 8 respectively. Serotypes 5 and 9 are not included in vaccine formulations. A monovalent type can also be prepared. After addition of suitable stabiliser, the vaccine is distributed in 1.0 ml volumes into glass vials and freeze-dried.

#### 3.2.4. Final product batch test

i) Sterility

Following lyophilisation, five bottles of vaccine are selected at random and tested for sterility by internationally accepted methods. Tests for sterility and freedom from contamination of biological materials intended for veterinary use are given in chapter 1.1.9.

ii) Safety

Innocuity of a vaccine is determined by the inoculation of reconstituted vaccine into mice (0.25 ml intraperitoneally), guinea-pig (1.0 ml intraperitoneally), and a horse (5.0 ml subcutaneously). All the animals are observed daily for 14 days. The rectal temperature of the horse is taken twice daily for 14 days and should never exceed 39°C.

iii) Batch potency

Potency is largely based on virus concentration in the vaccine.

The minimum immunising dose for each serotype is about $1 \times 10^3$ PFU/dose. The infectivity titre of the final product is assayed by plaque titration in Vero cell cultures and should contain at least $1 \times 10^5$ PFU/dose. The horse used for safety testing is also used for determining the immunogenicity of a vaccine.

Serum samples are collected on the day of vaccination and 21 days later, and are tested for neutralising antibodies against each serotype by the plaque-reduction test using twofold serum dilutions and about 100 PFU of virus. The horse should develop a neutralising antibody titre of at least 20 against at least three of the four serotypes in the quadrivalent vaccine.

### 3.3. Requirements for regulatory approval

No specific guideline is described for AHS vaccine. However, a guideline is described in the EU for Bluetongue virus under exceptional circumstances that could probably be used for AHS virus. This guideline includes the minimum date requirements for the regulatory approval under exceptional circumstances for vaccine production for emergency use against bluetongue virus (Regulation EC Nº726/2004, in particular Articles 38, 39 and 43 thereof and Article 26 of Direction 2001/82/EC), including guidance measures to facilitate the rapid inclusion of new or different virus serotypes.

## 4. Vaccines based on biotechnology

### 4.1. Vaccines available and their advantages

None is available commercially. Experimental vaccines developed by different approaches have been described (Section C.1.1 *Rationale and intended use of the product*).

### 4.2. Special requirements for biotechnological vaccines, if any

None.

## REFERENCES

AGÜERO M., GÓMEZ-TEJEDOR C., ANGELES CUBILLO M., RUBIO C., ROMERO E. & JIMÉNEZ-CLAVERO A. (2008). Real-time fluorogenic reverse transcription polymerase chain reaction assay for detection of African horse sickness virus. *J. Vet. Diagn. Invest.,* **20**, 325–328.

ALBERCA B., BACHANEK-BANKOWSKA K., CABANA M., CALVO-PINILLA E., VIAPLANA E., FROST L., GUBBINS S., URNIZA A., MERTENS P. & CASTILLO-OLIVARES J. (2014). Vaccination of horses with a recombinant modified vaccinia Ankara virus (MVA) expressing African horse sickness (AHS) virus major capsid protein VP2 provides complete clinical protection against challenge. *Vaccine*, **32**, 3670–3674.

ARADAIB I.E. (2009). PCR detection of African horse sickness virus serogroup based on genome segment three sequence analysis. *J. Virol. Methods*, **159**,1–5.

BACHANEK-BANKOWSKA K., MAAN S., CASTILLO-OLIVARES J., MANNING N.M., MAAN N.S., POTGIETER A.C., DI NARDO A., SUTTON G., BATTEN C. & MERTENS P.P. (2014). Real-time RT-PCR assays for detection and typing of African horse sickness virus. *PLoS One*, **9** (4), e93758.

BREMER C.W. & VILJOEN G.J. (1998) Detection of African horsesickness virus and discrimination between two equine orbivirus serogroups by reverse transcription polymerase chain reaction. *Onderstepoort J. Vet. Res.,* **65**, 1–8.

COETZER J.A.W. & GUTHRIE.A.J. (2005). African horsesickness. *In:* Infectious Diseases of Livestock, Second Edition. Coetzer J.A.W. & Tustin R.C., eds. Oxford University Press, Cape Town, 1231–1246.

DURÁN-FERRER M., AGÜERO M., ZIENTARA S., SMITH S., POTGIETER C., RUEDA P., SASTRE P., MONACO F., VILLALBA R., TENA-TOMÁS C., BATTEN C., FROST L., FLANNERY J., GUBBINS S., LUBISI B.A., SÁNCHEZ-VIZCAÍNO J.M., EMERY M., STURGILL T., OSTLUND E. & CASTILLO-OLIVARES J. (2019). Assessment of reproducibility of a VP7 Blocking ELISA diagnostic test for African horse sickness. *Transbound. Emerg. Dis.*, doi: 10.1111/tbed.12968.

GUTHRIE A.J., MACLACHLAN N.J., JOONE C., LOURENS C.W., WEYER C.T., QUAN M., MONYAI M.S. & GARDNER I.A. (2013). Diagnostic accuracy of a duplex real-time reverse transcription quantitative PCR assay for detection of African horsesickness virus. *J. Virol. Methods,* **189**, 30–35.

HOUSE C., MIKICIUK P.E. & BERNINGER M.L. (1990). Laboratory diagnosis of African horse sickness: comparison of serological techniques and evaluation of storage methods of samples for virus isolation. *J. Vet. Diagn. Invest.,* **2**, 44–50.

HOUSE J., LOMBARD M., HOUSE C., DUBOURGET P. & MEBUS C. (1992). Efficacy of an inactivated vaccine for African horse sickness serotype 4. *In:* Bluetongue, African Horse Sickness and Related Orbiviruses: Proceedings of the Second International Symposium, Walton T.E. & Osburn B.I., eds. CRC Press, Boca Raton, Florida, USA, 891–895.

KOEKEMOER J.J. (2008). Serotype-specific detection of African horsesickness virus by real-time PCR and the influence of genetic variations. *J. Virol. Methods*, **154**, 104–110.

LAVIADA M.D., ROY P. & SANCHEZ-VIZCAINO J.M (1992). Adaptation and evaluation of an indirect ELISA and inmunoblotting test for African horse sickness antibody detection. *In:* Bluetongue, African Horse Sickness and Related Orbiviruses: Proceedings of the Second International Symposium. Walton T.E. & Osburn B.I., Eds. CRC Press, Boca Raton, Florida, USA, 646–650.

LU G., PAN J., OU J., SHAO R., HU X., WANG C. & LI S. (2020). African horse sickness: Its emergence in Thailand and potential threat to other Asian countries. *Transbound. Emerg. Dis.,* **67**, 1751–1753. doi: 10.1111/tbed.13625.

MAAN N.S., MAAN S., NOMIKOU K., BELAGANAHALLI M.N., BACHANEK-BANKOWSKA K. & MERTENS P.P.C. (2011). Serotype-specific primers and gel-based RT-PCR assays for 'typing' African horse sickness virus: identification of strains from Africa. *PLoS One,* **6** (10), e25686.

MAREE S. & PAWESKA J.T. (2005). Preparation of recombinant African horse sickness virus VP7 antigen via a simple method and validation of a VP7-based indirect ELISA for the detection of group-specific IgG antibodies in horse sera. *J. Virol. Methods,* **125**, 55–65.

MARTINEZ J., DIAZ-LAVIADA M., ROY P., SANCHEZ C., VELA C., SANCHEZ-VIZCAINO J.M. & CASAL I. (1996). Full protection against AHSV in horses induced by baculovirus-derived AHS virus serotype 4 VP2, VP5 and VP7. *J. Gen. Virol.,* **77**, 1211–1221.

O'DELL N., ARNOT L. JANISCH C.E. & STEYL J.C.A. (2018). Clinical presentation and pathology of suspected vector-transmitted African horse sickness in South African domestic dogs from 2006 to 2017. *Vet. Rec.* **182**, 715. doi:10.1136/vr.104611

O'KENNEDY M.M., ROTH R., EBERSOHN K., DU PLESSIS L.H., MAMPUTHA S., RUTKOWSKA D.A., DU PREEZ I., VERSCHOOR J.A. & LEMMER Y. (2024). Immunogenic profile of a plant-produced nonavalent African horse sickness viral protein 2 (VP2) vaccine in IFNAR$^{-/-}$ mice. *PLoS One*, 19, e0301340.

Quan M., Lourens C.W., MacLachlan N.J., Gardner I.A. & Guthrie A.J. (2010). Development and optimisation of a duplex real-time reverse transcription quantitative PCR assay targeting the VP7 and NS2 genes of African horse sickness virus. *J. Virol. Methods*, **167**, 45–52.

Rodríguez M., Joseph S., Pfeffer M., Raghavan R. & Wernery U. (2020). Immune response of horses to inactivated African horse sickness vaccines. *BMC Vet. Res.*, **6**, 322. doi: 10.1186/s12917-020-02540-y.

Roy P., Mertens P.C. & Casal I. (1994). African Horse Sickness Structure. *Comp. Immunol. Microb. And Infec. Dis.*, **17**, 243–273.

Scanlen M., Paweska J., Verschoor J. & Dijk A. (2002). The protective efficacy of a recombinant VP2-based African horsesickness subunit vaccine candidate is determined by adjuvant. *Vaccine*, **20**, 1079–1088.

van der Meyden C.H., Erasmus B.J., Swanepoel R. & Prozesky O.W. (1992) Encephalitis and chorioretinitis associated with neurotropic African horsesickness virus infection in laboratory workers. Part I. Clinical and neurological observations. *S. Afr. Med. J.*, **81**, 451–454.

Van Rijn P.A., Maris-Veldhuis M.A., Potgieter C.A. & van Gennip R.G.P. (2018). African horse sickness virus (AHSV) with a deletion of 77 amino acids in NS3/NS3a protein is not virulent and a safe promising AHS Disabled Infectious Single Animal (DISA) vaccine platform. *Vaccine*, **36**, 1925–1933.

Van Schalkwyk A., Ferreira M.L. & Romito M. (2019). Using a new serotype-specific polymerase chain reaction (PCR) and sequencing to differentiate between field and vaccine-derived African horse sickness viruses. *J. Virol. Methods*, **266**, 89–94.

Verwoerd D.W., Huismans H., Erasmus B.J. (1979). Orbiviruses. *In:* Comprehensive Virology, Fraenkel-Conrat H., Wagner R.R., eds. Plenum Press, London, UK, Vol. **14**, 285–345.

Villalba R., Tena-Tomás C., Ruano M. J., Valero-Lorenzo M., López-Herranz A. & Agüero M. (2024). Development and validation of three triplex Real-Time RT-PCR assays for typing African Horse Sickness virus: utility for disease control and other laboratory applications. *Viruses*, **16**, 470.

Weyer C.T., Grewar J.D., Burger P., Joone C., Lourens C., MacLachlan N.J. & Guthrie A.J. (2017). Dynamics of African horse sickness virus nucleic acid and antibody in horses following immunization with a commercial polyvalent live attenuated vaccine. *Vaccine*, 35, 2504–2510.

Weyer C.T., Joone C., Lourens C.W., Monyai M.S., Koekemoer O., Grewar J.D., van Schalkwyk A., Majiwa P.O., MacLachlan N.J. & Guthrie A.J. (2015). Development of three triplex real-time reverse transcription PCR assays for the qualitative molecular typing of the nine serotypes of African horse sickness virus. *J. Virol. Methods*, **223**, 69–74.

World Organisation for Animal Health (WOAH) (2010). African horse sickness. *In:* Atlas of Transboundary Animal Diseases, Fernandez P.J. & White W.R., eds. WOAH, Paris, France, 12–18.

Zientara S., Sailleau C., Moulay S. & Cruciere C. (1994). Diagnosis of the African horse sickness virus serotype 4 by a one-tube, one manipulation RT-PCR reaction from infected organs. *J. Virol. Methods*, **46**, 179–188.

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**NB:** There are WOAH Reference Laboratories for African horse sickness (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 African horse sickness

***Appendix 1: African horse sickness***
***Intended purpose of test: population freedom from infection***

| Test with score and species | Sample type and target analytes | Accuracy | Test population | Validation report | Advantages | Disadvantages | References |
|---|---|---|---|---|---|---|---|

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Identification of the agent | | | | | | | |
| Real-time RT-PCR ☑□□ Equids | EDTA blood/tissue (VP7) | (1) Dse 97% DSp 100%<br><br>(2) Dse > 99.9% DSp > 97.8% | (1) 186 known negatives and 132 known positive samples<br><br>(2) 503 equine blood samples collected from suspect cases; two separate healthy populations of horses (503 and 98 horses) were used as negative cases | (1) Validation report done at LCV (EURL), sent to WOAH and published in WOAH *Bulletin* nº 2016-1<br><br>(2) Validation report done at CVR (WOAH-RL), sent to WOAH and published in Guthrie *et al.* (2013) | - High sensitivity, specificity and reproducibility<br>- High throughput<br>- Minimal risk of cross contamination<br>- Applicable to uninfectious samples allowing viral genome to be detected even after the viraemic window as well as in poor condition or inactivated samples | - Expensive equipment<br>- Expensive reagents<br>- Surviving animals present shorter periods of viraemia in comparison with antibodies<br>- Detection of viral genome in animals vaccinated with live attenuated AHS vaccines has been described up to 16 weeks after vaccination | (1) Agüero *et al.* (2008)<br>(2) Guthrie *et al.* (2013)<br>Quan *et al.* (2010) |
| Agarose gel-based RT-PCR ☑□□ | EDTA blood/tissue (VP7) | Not available | Not available | | - Less expensive instruments than those used in real-time RT-PCR<br>- High sensitivity, specificity and reproducibility<br>- Applicable to uninfectious samples | - Longer analysis time than real-time RT-PCR<br>- Not suitable for high throughput analysis<br>- Risk of cross contamination<br>-Surviving animals present shorter periods of viraemia in comparison with antibodies | Zientara *et al.* (1994) |
| Virus isolation □□□ | EDTA blood/tissue | DSe ≥ 50% DSp > 99% | - 91 BTV positive blood samples collected from naturally infected animals during BTV outbreaks in Spain in 2007–2018 (validation done jointly for all Orbiviruses)<br>- 12 tissue samples from horses experimentally infected with AHSV-9 | Dossier for Verification of the Orbivirus isolation carried out at LCV (EURL) | - Detection of infectious virus<br>- Getting available isolates for further studies | - Time consuming<br>- Laborious<br>- Requirement for samples from viraemic period<br>- Requirement for well conserved samples<br>- Biosafety laboratory consideration BLS3<br>- Requirement for cell cultures<br>- Low sensitivity | |
| Detection of immune response | | | | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| ELISA (serogroup specific based on VP7) ☑☑☑ Equids | Serum (antibodies against VP7) | (1) DSe ≥ 95% DSp > 99%<br><br>(2) DSe > 99% Dsp > 99% | (1, 2) Goat, sheep and guinea-pig sera from animals inoculated with AHS1-AHS9<br>(1) Nine sera from horse naturally infected and positive by VN<br>(1) 152 sera from horses vaccinated and serotyped by VN<br>(1, 2) Sera from horses from free areas (512, 1015)<br>(1) 24 bovine pos to BTV, 20 horse sera pos to EA and nine deer sera pos to EHDV<br>(1) Seven sera from the WOAH reference collection, from different equidae species and in different levels of positivity<br>(1) Seven sera from two experimentally infected horses with AHS9<br>(1) 70 samples from the EU-RL proficiency tests organised from 2014 to 2020<br>(1) 129 pos sera from vaccinated or experimentally or naturally infected Equidae, and 57 neg sera from naïve equidae | (1, 2) Manufacturer's validated method certificates<br>(1) Dossier for Verification of blocking ELISA for detection of antibodies against African horse sickness virus carried out at LCV (EU-RL). Stage 3 of validation (PT) published in Durán-Ferrer *et al.* (2019) | - High sensitivity and specificity<br>- Commercially available (both B-ELISA and I-ELISA)<br>- Cost-effective<br>- VP7 is highly conserved among serotypes<br>-Long lasting of presence of antibodies in affected animals | - Unable to differentiate vaccinated from infected animals<br>- Unable to differentiate serotype specificity of the antibodies | (1) B-ELISA INGEZIM AHSV Compac Plus 2.0 (Gold Standard Diagnostics); Durán-Ferrer *et al.* (2019)<br>(2) I-ELISA IDScreen African horse sickness indirect (IDVET) |
| VN ☑☐☐ Equids | Serum (neutralising serotype-specific antibodies against VP2) | DSe: > 95% (infected animals) DSp > 99% | (1) 18 sera from sheep experimentally inoculated with AHS1–AHS9<br>(2) Seven sera from two experimentally infected horses with AHS9 | Dossier for Verification of the Orbivirus Seroneutralisation Test carried out at LCV (EU-RL) | - Allows serotyping from serum samples | - Unable to differentiate vaccinated from infected animals<br>- Depends on the availability of live virus strains of all serotypes and susceptible cultured cells<br>- Cross-reactions may appear between serotypes<br>- Time consuming<br>- Laborious<br>- Biosafety laboratory consideration BLS3 | |

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|---|---|---|---|---|---|---|---|
| CFT ☑□□ Equids | Serum | Not available | Not available | Not available | - Useful in recent infections or vaccination | - High number of sera presenting anticomplementary activity<br>-Less sensitivity than ELISA<br>-Less degree of standardisation than ELISA | |

***Appendix 2: African horse sickness***

***Intended purpose of test: individual animal freedom from infection prior to movement****

| Test with score and species | Sample type and target analytes | Accuracy | Test population | Validation report | Advantages | Disadvantages | References |
|---|---|---|---|---|---|---|---|

| Identification of the agent | | | | | | | |
|---|---|---|---|---|---|---|---|
| Real-time RT-PCR ☑☑☑ Equids | EDTA blood/tissue (VP7) | (1) Dse 97% DSp 100%<br><br>(2) Dse > 99.9% DSp > 97.8% | (1) 186 known negatives and 132 known positive samples<br><br>(2) 503 equine blood samples collected from suspect cases; two separate healthy populations of horses (503 and 98 horses) were used as negative cases | (1) Validation report done at LCV (EURL), sent to WOAH and published in WOAH *Bulletin* nº 2016-1<br><br>(2) Validation report done at CVR (WOAH-RL), sent to WOAH and published in Guthrie *et al.* (2013) | - High sensitivity, specificity and reproducibility<br>- Rapid technique<br>- Minimal risk of cross contamination<br>- Applicable to uninfectious samples allowing viral genome to be detected even after the viraemic window as well as in poor condition or inactivated samples | - Expensive equipment<br>- Expensive reagents<br>- Surviving animals present shorter periods of viraemia in comparison with antibodies<br>- Detection of viral genome in animals vaccinated with live attenuated AHS vaccines has been described up to 16 weeks after vaccination<br>- Discordant results are possible in the case of weak positive samples | (1) Agüero *et al.* (2008)<br>(2) Guthrie *et al.* (2013)<br>Quan *et al.,* (2010) |
| Agarose gel-based RT-PCR ☑☑□ | EDTA blood/tissue (VP7) | Not available | Not available | | - Less expensive instruments than those used in real-time RT-PCR<br>- High sensitivity, specificity and reproducibility<br>- Rapid technique<br>- Applicable to uninfectious samples | - Longer analysis time than real-time RT-PCR<br>- Risk of cross contamination<br>-Surviving animals present shorter periods of viraemia in comparison with antibodies | Zientara *et al.* (1994) |
| Virus isolation – ☑□□ | EDTA blood/tissue | DSe ≥ 50%<br>DSp > 99% | - 91 BTV positive blood samples collected from naturally infected animals during BTV outbreaks in Spain in 2007–2018 (validation done jointly for all Orbiviruses)<br>- 12 tissue samples from horses experimentally infected with AHSV-9 | Dossier for Verification of the Orbivirus isolation carried out at LCV (EURL) | - Detection of infectious virus | - Time consuming<br>- Laborious<br>- Requirement for well conserved samples<br>- Biosafety laboratory consideration BLS3<br>- Requirement for cell cultures<br>- Low sensitivity | |
| *Detection of immune response | | | | | | | |

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|---|---|---|---|---|---|---|---|
| ELISA (serogroup specific based on VP7) ☑☑☑ Equids | Serum (antibodies against VP7) | (1) DSe ≥ 95% DSp > 99%<br><br>(2) DSe > 99% Dsp > 99% | (1, 2) Goat, sheep and guinea-pig sera from animals inoculated with AHS1-AHS9<br>(1) Nine sera from horse naturally infected and positive by VN<br>(1) 152 sera from horses vaccinated and serotyped by VN<br>(1, 2) Sera from horses from free areas (512, 1015)<br>(1) 24 bovine pos to BTV, 20 horse sera pos to EA and nine deer sera pos to EHDV<br>(1) Seven sera from the WOAH reference collection, from different equidae species and in different levels of positivity<br>(1) Seven sera from two experimentally infected horses with AHS9<br>(1) 70 samples from the EU-RL proficiency tests organised from 2014 to 2020<br>(1) 129 pos sera from vaccinated or experimentally or naturally infected equidae, and 57 neg sera from naïve equidae | (1, 2) Manufacturer's validated method certificates<br>(1) Dossier for Verification of blocking ELISA for detection of antibodies against African horse sickness virus carried out at LCV (EU-RL). Stage 3 of validation (PT) published in Durán-Ferrer *et al.* (2019) | - High sensitivity and specificity<br>- Commercially available (both B-ELISA and I-ELISA)<br>- Cost-effective<br>- VP7 is highly conserved among serotypes<br>-Long lasting of presence of antibodies in affected animals | - Unable to differentiate vaccinated from infected animals<br>- Seroconversion takes place after at least 8–12 d.p.i. | (1) B-ELISA INGEZIM AHSV Compac Plus 2.0 (Gold Standard Diagnostics); Durán-Ferrer *et al.* (2019)<br>(2) I-ELISA IDScreen African horse sickness indirect (IDVET) |
| VN ☑☐☐ Equids | Serum (neutralising serotype-specific antibodies against VP2) | DSe: > 95% (infected animals) DSp > 99% | (1) 18 sera from sheep experimentally inoculated with AHS1–AHS9<br>(2) Seven sera from two experimentally infected horses with AHS9 | Dossier for Verification of the Orbivirus Seroneutralisation Test carried out at LCV (EU-RL) | - Allows serotyping from serum samples | - Unable to differentiate vaccinated from infected animals<br>- Depends on the availability of live virus strains of all serotypes and susceptible cultured cells<br>- Cross-reactions may appear between serotypes<br>- Time consuming<br>- Laborious<br>- Biosafety laboratory consideration BLS3 | |

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|---|---|---|---|---|---|---|---|
| CFT ☑□□ Equids | Serum | Not available | Not available | Not available | - Useful in recent infections or vaccination | - High number of sera presenting anticomplementary activity<br>-Less sensitivity than ELISA<br>-Less degree of standardisation than ELISA | |

*For serological tests, paired samples may be required as recommended in the *Terrestrial Code* Chapter 12.1. 'Infection with African horse sickness virus', Article 12.1.7 'Recommendations for importation from AHS infected countries or zones'

***Appendix 3: African horse sickness***

***Intended purpose of test: contribute to eradication policies***

| Test with score and species | Sample type and target analytes | Accuracy | Test population | Validation report | Advantages | Disadvantages | References |
|---|---|---|---|---|---|---|---|

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Identification of the agent | | | | | | | |
| Real-time RT-PCR ☑☑☑ Equids | EDTA blood/tissue (VP7) | (1) Dse 97% DSp 100%<br><br>(2) Dse > 99.9% DSp > 97.8% | (1) 186 known negatives and 132 known positive samples<br><br>(2) 503 equine blood samples collected from suspect cases; two separate healthy populations of horses (503 and 98 horses) were used as negative cases | (1) Validation report done at LCV (EURL), sent to WOAH and published in WOAH *Bulletin* nº 2016-1<br><br>(2) Validation report done at CVR (WOAH-RL), sent to WOAH and published in Guthrie *et al.* (2013) | - High sensitivity, specificity and reproducibility<br>- High throughput<br>- Rapid technique<br>- Minimal risk of cross contamination<br>- Applicable to uninfectious samples allowing viral genome to be detected even after the viraemic window as well as in poor condition or inactivated samples | - Expensive equipment<br>- Expensive reagents<br>- Surviving animals present shorter periods of viraemia in comparison with antibodies<br>- Detection of viral genome in animals vaccinated with live attenuated AHS vaccines has been described up to 16 weeks after vaccination | (1) Agüero *et al.* (2008)<br>(2) Guthrie *et al.* (2013)<br>Quan *et al.,* (2010) |
| Agarose gel-based RT-PCR ☑☑□ | EDTA blood/tissue (VP7) | Not available | Not available | | - Less expensive instruments than those used in real-time RT-PCR<br>- High sensitivity, specificity and reproducibility<br>- Rapid technique<br>- Applicable to uninfectious samples | - Longer analysis time than real-time RT-PCR<br>- Not suitable for high throughput analysis<br>- Risk of cross contamination<br>-Surviving animals present shorter periods of viraemia in comparison with antibodies | Zientara *et al.* (1994) |
| Virus isolation □□□ | EDTA blood/tissue | DSe ≥ 50% DSp > 99% | - 91 BTV positive blood samples collected from naturally infected animals during BTV outbreaks in Spain in 2007–2018 (validation done jointly for all Orbiviruses)<br>- 12 tissue samples from horses experimentally infected with AHSV-9 | Dossier for Verification of the Orbivirus isolation carried out at LCV (EURL) | - Detection of infectious virus<br>- Getting available isolates for further studies | - Time consuming<br>- Laborious<br>- Requirement for samples from viraemic period<br>- Requirement for well conserved samples<br>- Biosafety laboratory consideration BLS3<br>- Requirement for cell cultures<br>- Low sensitivity | |
| Detection of immune response | | | | | | | |

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|---|---|---|---|---|---|---|---|
| ELISA (serogroup specific based on VP7) ☑☑☐ Equids | Serum (antibodies against VP7) | (1) DSe ≥ 95% DSp > 99%<br><br>(2) DSe > 99% Dsp > 99% | (1, 2) Goat, sheep and guinea-pig sera from animals inoculated with AHS1-AHS9<br>(1) Nine sera from horse naturally infected and positive by VN<br>(1) 152 sera from horses vaccinated and serotyped by VN<br>(1, 2) Sera from horses from free areas (512, 1015)<br>(1) 24 bovine pos to BTV, 20 horse sera pos to EA and nine deer sera pos to EHDV<br>(1) Seven sera from the WOAH reference collection, from different equidae species and in different levels of positivity<br>(1) Seven sera from two experimentally infected horses with AHS9<br>(1) 70 samples from the EU-RL proficiency tests organised from 2014 to 2020<br>(1) 129 pos sera from vaccinated or experimentally or naturally infected equidae, and 57 neg sera from naïve equidae | (1, 2) Manufacturer's validated method certificates<br>(1) Dossier for Verification of blocking ELISA for detection of antibodies against African horse sickness virus carried out at LCV (EU-RL). Stage 3 of validation (PT) published in Durán-Ferrer *et al.* (2019) | - High sensitivity and specificity<br>- Commercially available (both B-ELISA and I-ELISA)<br>- Cost-effective<br>- VP7 is highly conserved among serotypes<br>-Long lasting of presence of antibodies in affected animals | - Unable to differentiate vaccinated from infected animals<br>- Unable to differentiate serotype specificity of the antibodies<br>- Seroconversion takes place after at least 8–12 d.p.i. | (1) B-ELISA INGEZIM AHSV Compac Plus 2.0 (Gold Standard Diagnostics); Durán-Ferrer *et al.* (2019)<br>(2) I-ELISA IDScreen African horse sickness indirect (IDVET) |
| VN □□□ Equids | Serum (neutralising serotype-specific antibodies against VP2) | DSe: > 95% (infected animals) DSp > 99% | (1) 18 sera from sheep experimentally inoculated with AHS1–AHS9<br>(2) Seven sera from two experimentally infected horses with AHS9 | Dossier for Verification of the Orbivirus Seroneutralisation Test carried out at LCV (EU-RL) | - Allows serotyping from serum samples | - Unable to differentiate vaccinated from infected animals<br>- Depends on the availability of live virus strains of all serotypes and susceptible cultured cells<br>- Cross-reactions may appear between serotypes<br>- Time consuming<br>- Laborious<br>- Biosafety laboratory consideration BLS3 | |

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|---|---|---|---|---|---|---|---|
| CFT ☑□□ Equids | Serum | Not available | Not available | Not available | - Useful in recent infections or vaccination | - High number of sera presenting anticomplementary activity<br>-Less sensitivity than ELISA<br>-Less degree of standardisation than ELISA | |

***Appendix 4: African horse sickness***

***Intended purpose of test: confirmation of clinical cases***

| Test with score and species | Sample type and target analytes | Accuracy | Test population | Validation report | Advantages | Disadvantages | References |
|---|---|---|---|---|---|---|---|

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|---|---|---|---|---|---|---|---|
| Identification of the agent | | | | | | | |
| Real-time RT-PCR ☑☑☑ Equids | EDTA blood/tissue (VP7) | (1) Dse 97% DSp 100%<br><br>(2) Dse > 99.9% DSp > 97.8% | (1) 186 known negatives and 132 known positive samples<br><br>(2) 503 equine blood samples collected from suspect cases; two separate healthy populations of horses (503 and 98 horses) were used as negative cases | (1) Validation report done at LCV (EURL), sent to WOAH and published in WOAH *Bulletin* nº 2016-1<br><br>(2) Validation report done at CVR (WOAH-RL), sent to WOAH and published in Guthrie *et al.* (2013) | - High sensitivity, specificity and reproducibility<br>- High throughput<br>- Rapid technique<br>- Minimal risk of cross contamination<br>- Applicable to uninfectious samples allowing viral genome to be detected even after the viraemic window as well as in poor condition or inactivated samples | - Expensive equipment<br>- Expensive reagents<br>- Surviving animals present shorter periods of viraemia in comparison with antibodies<br>- Detection of viral genome in animals vaccinated with live attenuated AHS vaccines has been described up to 16 weeks after vaccination | (1) Agüero *et al.* (2008)<br>(2) Guthrie *et al.* (2013)<br>Quan *et al.*, (2010) |
| Agarose gel-based RT-PCR ☑☑☐ | EDTA blood/tissue (VP7) | Not available | Not available | | - Less expensive instruments than those used in real-time RT-PCR<br>- High sensitivity, specificity and reproducibility<br>- Rapid technique<br>- Applicable to uninfectious samples | - Longer analysis time than real-time RT-PCR<br>- Not suitable for high throughput analysis<br>- Risk of cross contamination<br>-Surviving animals present shorter periods of viraemia in comparison with antibodies | Zientara *et al.* (1994) |
| Virus isolation ☑☑☑ | EDTA blood/tissue | DSe ≥ 50% DSp > 99% | - 91 BTV positive blood samples collected from naturally infected animals during BTV outbreaks in Spain in 2007–2018 (validation done jointly for all Orbiviruses)<br>- 12 tissue samples from horses experimentally infected with AHSV-9 | Dossier for Verification of the Orbivirus isolation carried out at LCV (EURL) | - Detection of infectious virus<br>- Getting available isolates for further studies | - Time consuming<br>- Laborious<br>- Requirement for samples from viraemic period<br>- Requirement for well conserved samples<br>- Biosafety laboratory consideration BLS3<br>- Requirement for cell cultures<br>- Low sensitivity | |
| Detection of immune response | | | | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| ELISA (serogroup specific based on VP7) ☑□□ Equids | Serum (antibodies against VP7) | (1) DSe ≥ 95% DSp > 99%<br><br>(2) DSe > 99% Dsp > 99% | (1, 2) Goat, sheep and guinea-pig sera from animals inoculated with AHS1-AHS9<br>(1) Nine sera from horse naturally infected and positive by VN<br>(1) 152 sera from horses vaccinated and serotyped by VN<br>(1, 2) Sera from horses from free areas (512, 1015)<br>(1) 24 bovine pos to BTV, 20 horse sera pos to EA and nine deer sera pos to EHDV<br>(1) Seven sera from the WOAH reference collection, from different equidae species and in different levels of positivity<br>(1) Seven sera from two experimentally infected horses with AHS9<br>(1) 70 samples from the EU-RL proficiency tests organised from 2014 to 2020<br>(1) 129 pos sera from vaccinated or experimentally or naturally infected equidae, and 57 neg sera from naïve equidae | (1, 2) Manufacturer's validated method certificates<br>(1) Dossier for Verification of blocking ELISA for detection of antibodies against African horse sickness virus carried out at LCV (EU-RL). Stage 3 of validation (PT) published in Durán-Ferrer *et al.* (2019) | - High sensitivity and specificity<br>- Commercially available (both B-ELISA and I-ELISA)<br>- Cost-effective<br>- VP7 is highly conserved among serotypes | - Unable to differentiate vaccinated from infected animals<br>- Unable to differentiate serotype specificity of the antibodies<br>- Seroconversion takes place after at least 8–12 d.p.i. | (1) B-ELISA INGEZIM AHSV Compac Plus 2.0 (Gold Standard Diagnostics); Durán-Ferrer *et al.* (2019)<br>(2) I-ELISA IDScreen African horse sickness indirect (IDVET) |
| VN ☑□□ Equids | Serum (neutralising serotype-specific antibodies against VP2) | DSe: > 95% (infected animals) DSp > 99% | (1) 18 sera from sheep experimentally inoculated with AHS1–AHS9<br>(2) Seven sera from two experimentally infected horses with AHS9 | Dossier for Verification of the Orbivirus Seroneutralisation Test carried out at LCV (EU-RL) | - Allows serotyping from serum samples | - Unable to differentiate vaccinated from infected animals<br>- Depends on the availability of live virus strains of all serotypes and susceptible cultured cells<br>- Cross-reactions may appear between serotypes<br>- Time consuming<br>- Laborious<br>- Biosafety laboratory consideration BLS3 | |

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|---|---|---|---|---|---|---|---|
| CFT ☑□□ Equids | Serum | Not available | Not available | Not available | - Useful in recent infections or vaccination | - High number of sera presenting anticomplementary activity<br>-Less sensitivity than ELISA<br>-Less degree of standardisation than ELISA | |

***Appendix 5: African horse sickness***

***Intended purpose of test: prevalence of infection – surveillance***

| Test with score and species | Sample type and target analytes | Accuracy | Test population | Validation report | Advantages | Disadvantages | References |
|---|---|---|---|---|---|---|---|

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|---|---|---|---|---|---|---|---|
| Identification of the agent | | | | | | | |
| Real-time RT-PCR ☑☑□ Equids | EDTA blood/tissue (VP7) | (1) Dse 97% DSp 100%<br><br>(2) Dse > 99.9% DSp > 97.8% | (1) 186 known negatives and 132 known positive samples<br><br>(2) 503 equine blood samples collected from suspect cases; two separate healthy populations of horses (503 and 98 horses) were used as negative cases | (1) Validation report done at LCV (EURL), sent to WOAH and published in WOAH *Bulletin* nº 2016-1<br><br>(2) Validation report done at CVR (WOAH-RL), sent to WOAH and published in Guthrie *et al.* (2013) | - High sensitivity, specificity and reproducibility<br>- High throughput<br>- Rapid technique<br>- Minimal risk of cross contamination<br>- Applicable to uninfectious samples allowing viral genome to be detected even after the viraemic window as well as in poor condition or inactivated samples | - Expensive equipment<br>- Expensive reagents<br>- Surviving animals present shorter periods of viraemia in comparison with antibodies<br>- Detection of viral genome in animals vaccinated with live attenuated AHS vaccines has been described up to 16 weeks after vaccination | (1) Agüero *et al.* (2008)<br>(2) Guthrie *et al.* (2013)<br>Quan *et al.,* (2010) |
| Agarose gel-based RT-PCR ☑□□ | EDTA blood/tissue (VP7) | Not available | Not available | | - Less expensive instruments than those used in real-time RT-PCR<br>- High sensitivity, specificity and reproducibility<br>- Rapid technique<br>- Applicable to uninfectious samples | - Longer analysis time than real-time RT-PCR<br>- Not suitable for high throughput analysis<br>- Risk of cross contamination<br>-Surviving animals present shorter periods of viraemia in comparison with antibodies | Zientara *et al.* (1994) |
| Virus isolation □□□ | EDTA blood/tissue | DSe ≥ 50% DSp > 99% | - 91 BTV positive blood samples collected from naturally infected animals during BTV outbreaks in Spain in 2007–2018 (validation done jointly for all Orbiviruses)<br>- 12 tissue samples from horses experimentally infected with AHSV-9 | Dossier for Verification of the Orbivirus isolation carried out at LCV (EURL) | - Detection of infectious virus<br>- Getting available isolates for further studies | - Time consuming<br>- Laborious<br>- Requirement for samples from viraemic period<br>- Requirement for well conserved samples<br>- Biosafety laboratory consideration BLS3<br>- Requirement for cell cultures<br>- Low sensitivity | |
| Detection of immune response | | | | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| ELISA (serogroup specific based on VP7) ☑☑☑ Equids | Serum (antibodies against VP7) | (1) DSe ≥ 95% DSp > 99%<br><br>(2) DSe > 99% Dsp > 99% | (1, 2) Goat, sheep and guinea-pig sera from animals inoculated with AHS1-AHS9<br>(1) Nine sera from horse naturally infected and positive by VN<br>(1) 152 sera from horses vaccinated and serotyped by VN<br>(1, 2) Sera from horses from free areas (512, 1015)<br>(1) 24 bovine pos to BTV, 20 horse sera pos to EA and nine deer sera pos to EHDV<br>(1) Seven sera from the WOAH reference collection, from different equidae species and in different levels of positivity<br>(1) Seven sera from two experimentally infected horses with AHS9<br>(1) 70 samples from the EU-RL proficiency tests organised from 2014 to 2020<br>(1) 129 pos sera from vaccinated or experimentally or naturally infected equidae, and 57 neg sera from naïve equidae | (1, 2) Manufacturer's validated method certificates<br>(1) Dossier for Verification of blocking ELISA for detection of antibodies against African horse sickness virus carried out at LCV (EU-RL). Stage 3 of validation (PT) published in Durán-Ferrer *et al.* (2019) | - High sensitivity and specificity<br>- Commercially available (both B-ELISA and I-ELISA)<br>- Cost-effective<br>- VP7 is highly conserved among serotypes | - Unable to differentiate vaccinated from infected animals<br>- Unable to differentiate serotype specificity of the antibodies<br>- Seroconversion takes place after at least 8–12 d.p.i. | (1) B-ELISA INGEZIM AHSV Compac Plus 2.0 (Gold Standard Diagnostics); Durán-Ferrer *et al.* (2019)<br>(2) I-ELISA IDScreen African horse sickness indirect (IDVET) |
| VN ☑☐☐ Equids | Serum (neutralising serotype-specific antibodies against VP2) | DSe: > 95% (infected animals) DSp > 99% | (1) 18 sera from sheep experimentally inoculated with AHS1–AHS9<br>(2) Seven sera from two experimentally infected horses with AHS9 | Dossier for Verification of the Orbivirus Seroneutralisation Test carried out at LCV (EU-RL) | - Allows serotyping from serum samples | - Unable to differentiate vaccinated from infected animals<br>- Depends on the availability of live virus strains of all serotypes and susceptible cultured cells<br>- Cross-reactions may appear between serotypes<br>- Time consuming<br>- Laborious<br>- Biosafety laboratory consideration BLS3 | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| CFT ☑□□ Equids | Serum | Not available | Not available | Not available | - Useful in recent infections or vaccination | - High number of sera presenting anticomplementary activity<br>-Less sensitivity than ELISA<br>-Less degree of standardisation than ELISA | |

***Appendix 6: African horse sickness***

***Intended purpose of test: immune status in individual animals or populations post-vaccination***

| Test with score and species | Sample type and target analytes | Accuracy | Test population | Validation report | Advantages | Disadvantages | References |
|---|---|---|---|---|---|---|---|

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Identification of the agent | | | | | | | |
| Real-time RT-PCR □□□ Equids | EDTA blood/tissue (VP7) | (1) Dse 97% DSp 100%<br><br>(2) Dse > 99.9% DSp > 97.8% | (1) 186 known negatives and 132 known positive samples<br><br>(2) 503 equine blood samples collected from suspect cases; two separate healthy populations of horses (503 and 98 horses) were used as negative cases | (1) Validation report done at LCV (EURL), sent to WOAH and published in WOAH *Bulletin* nº 2016-1<br><br>(2) Validation report done at CVR (WOAH-RL), sent to WOAH and published in Guthrie *et al.* (2013) | - None for this purpose | - Not suitable for this purpose | (1) Agüero *et al.* (2008)<br>(2) Guthrie *et al.* (2013)<br>Quan *et al.*, (2010) |
| Agarose gel-based RT-PCR □□□ | EDTA blood/tissue (VP7) | Not available | Not available | | - None for this purpose | - Not suitable for this purpose | Zientara *et al.* (1994) |
| Virus isolation □□□ | EDTA blood/tissue | DSe ≥ 50% DSp > 99% | - 91 BTV positive blood samples collected from naturally infected animals during BTV outbreaks in Spain in 2007–2018 (validation done jointly for all Orbiviruses)<br>- 12 tissue samples from horses experimentally infected with AHSV-9 | Dossier for Verification of the Orbivirus isolation carried out at LCV (EURL) | - None for this purpose | - Not suitable for this purpose | |
| Detection of immune response | | | | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| ELISA (serogroup specific based on VP7) ☑☑☐ Equids | Serum (antibodies against VP7) | (1) DSe ≥ 95% DSp > 99%<br><br>(2) DSe > 99% Dsp > 99% | (1, 2) Goat, sheep and guinea-pig sera from animals inoculated with AHS1-AHS9<br>(1) Nine sera from horse naturally infected and positive by VN<br>(1) 152 sera from horses vaccinated and serotyped by VN<br>(1, 2) Sera from horses from free areas (512, 1015)<br>(1) 24 bovine pos to BTV, 20 horse sera pos to EA and nine deer sera pos to EHDV<br>(1) Seven sera from the WOAH reference collection, from different Equidae species and in different levels of positivity<br>(1) Seven sera from two experimentally infected horses with AHS9<br>(1) 70 samples from the EU-RL proficiency tests organised from 2014 to 2020<br>(1) 129 pos sera from vaccinated or experimentally or naturally infected Equidae, and 57 neg sera from naïve, equidae | (1, 2) Manufacturer's validated method certificates<br>(1) Dossier for Verification of blocking ELISA for detection of antibodies against African horse sickness virus carried out at LCV (EU-RL). Stage 3 of validation (PT) published in Durán-Ferrer *et al.* (2019) | - High sensitivity and specificity<br>- Commercially available (both B-ELISA and I-ELISA)<br>- Cost-effective<br>- VP7 is highly conserved among serotypes<br>-Long lasting of presence of antibodies in affected animals | - Unable to differentiate serotype specificity of the antibodies<br>- Seroconversion takes place after at least 8–12 d.p.i. | (1) B-ELISA INGEZIM AHSV Compac Plus 2.0 (Gold Standard Diagnostics); Durán-Ferrer *et al.* (2019)<br>(2) I-ELISA IDScreen African horse sickness indirect (IDVET) |
| VN ☑☑☑ Equids | Serum (neutralising serotype-specific antibodies against VP2) | DSe: > 95% (infected animals) DSp > 99% | (1) 18 sera from sheep experimentally inoculated with AHS1–AHS9<br>(2) Seven sera from two experimentally infected horses with AHS9 | Dossier for Verification of the Orbivirus Seroneutralisation Test carried out at LCV (EU-RL) | - Allows serotyping from serum samples | - Depends on the availability of live virus strains of all serotypes and susceptible cultured cells<br>- Cross-reactions may appear between serotypes<br>- Time consuming<br>- Laborious<br>- Biosafety laboratory consideration BLS3 | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| CFT ☑□□ Equids | Serum | Not available | Not available | Not available | - Useful in recent infections or vaccination | - High number of sera presenting anticomplementary activity<br>-Less sensitivity than ELISA<br>-Less degree of standardisation than ELISA | |

[1] 20.5 g dextrose (114 mM), 7.9 g sodium citrate $2H_2O$ (27 mM), 4.2 g NaCl (71 mM), $H_2O$ to 1 litre. Adjust to pH with 1 M citric acid.

NB: First adopted in 1991. Most recent updates adopted in 2025.