SECTION 2.5.

EQUIDAE

CHAPTER 2.5.1.

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 affecting all species of equidae caused by an* Orbivirus *of the family* Reoviridae *and characterised by alterations in the respiratory and circulatory functions. AHS is transmitted by at least two species of* Culicoides. *Nine different serotypes have been described.*

*All serotypes of AHS occur in eastern and southern Africa. AHS serotypes 9, 4 and 2 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 (*1959–1963*), 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, they can 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.*

Identification of the agent: *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*), intravenously in embryonated eggs. Several enzyme-linked immunosorbent assays (*ELISAs*) for the rapid detection of AHSV antigen in blood, spleen tissues and supernatant from infected cells have been developed. Identification of AHSV RNA has also been achieved using a reverse-transcription polymerase chain reaction (*PCR*) method. 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.*

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 is used for serotyping.*

Requirements for vaccines: *Attenuated (*monovalent and polyvalent*) live vaccines for use in horses, mules and donkeys, are currently commercially available. Subunit vaccines have been evaluated experimentally.*

A. INTRODUCTION

African horse sickness (AHS) (Peste equina africana, Peste equine) is an infectious, non-contagious arthopod-borne disease of equidae, caused by a double-stranded RNA *Orbivirus* belonging to the family *Reoviridae*. The genus *Orbivirus* also includes bluetongue virus and epizootic haemorrhagic disease virus, 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 virion is an unenveloped particle of a size around 70 nm. The genome of AHS virus (AHSV) is composed of ten double-stranded RNA segments, encoding seven structural proteins (VP1-7), most of which have been completely sequenced for AHSV serotypes 4, 6 and 9 (Roy *et al.*, 1991; Venter *et al.*, 2000; Williams *et al.*, 1998), and four nonstructural proteins (NS1, NS2, NS3, NS3A) (Grubman & Lewis, 1992; Laviada *et al.*, 1993). 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. The NS3 proteins are the second most variable AHSV proteins (Van Niekert *et al.*, 2001), 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 (Martinez-Torrecuadrada *et al.*, 2001). 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), and in Europe (Spain: 1966, 1987–1990, and Portugal: 1989) (Sanchez-Vizcaíno, 2004).

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 resistant equidae such as zebra and donkeys (Coetzer & Guthrie, 2005).

The disease has both a seasonal (late summer/autumn) and a cyclical incidence with major epizootics in southern Africa during warm-phase events (Baylis *et al.*, 1999). Mortality due to AHS is related to the species of equidae affected 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 fever, and may have extended viraemia (up to 40 days).

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 (OIE, 2010).

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

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. 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*).

B. DIAGNOSTIC TECHNIQUES

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

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification[1]						
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 method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose; n/a = not applicable.
Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
RT-PCR = reverse-transcription polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay;
CFT = complement fixation test; VN = virus neutralisation.

Several techniques are already available for AHS viral identification ranging from the rapid capture (indirect sandwich) enzyme-linked immunosorbent assay (ELISA), using either polyclonal antibodies (PAbs) or monoclonal antibodies (MAbs), to the polymerase chain reaction (PCR) test, including reverse-transcription (RT) PCR for discrimination of the nine AHSV serotypes or virus isolation either in cell culture. 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 quick test such as ELISA or PCR, followed by virus isolation in tissue culture. Virus neutralisation (VN) for serotype identification, type-specific 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, there are no international standards for viruses or diagnostic reagents, and there is no standard methodology for the identification of AHSV. However, a viral and antibody panel has been evaluated, and comparative studies between different ELISAs for AHSV antigen and antibody determination have been carried out in different laboratories, including in the European Union (EU) Reference Laboratory for AHS. The results have demonstrated a high level of correlation for both antigen and antibody determination with an in-house test and commercial kits. Similar studies have been conducted with several RT-PCR assays also providing a high level of correlation. Further information on comparative studies of different test methods and kits is available from the OIE Reference Laboratories for AHS. A very important aspect of the diagnosis is the selection of samples and their safe transportation to the laboratory.

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

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 undiluted 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.

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. This technique has greatly improved the laboratory diagnosis of AHS by increasing the sensitivity of detection and shortening the time required for the diagnosis. 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. Therefore, positive results do not necessarily indicate the presence of infectious virus.

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 *et al.*, 1998; Laviada *et al.*, 1997; Sakamoto *et al.*, 1994; Stone-Marschat *et al.*, 1994; 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; Fernández-Pinero *et al.*, 2009; Rodriguez-Sanchez *et al.*, 2008). A duplex real-time RT-PCR has also been described that targets segments 7 and 8 of the genome (coding for NS1 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 (Laviada *et al.*, 1997; 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 OIE 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.

1.2.2. Agarose gel-based RT-PCR procedure (Laviada *et al.,* 1997; Zientara *et al.,* 1994)

Denaturation of extracted RNA has to be performed prior to the RT-PCR procedure as the AHSV genome consists of double-stranded RNA. The sequences of the PCR primers used are 5'-GTT-AAA-ATT-CGG-TTA-GGA-TG-3', which corresponds to the messenger RNA polarity, and 5'-GTA-AGT-GTA-TTC-GGT-ATT-G-3', which is complementary to the messenger RNA polarity.

All the components required for the reverse transcription and PCR are included in the reaction tube containing the denatured RNA. A one-step RT-PCR is carried out by incubating in a thermocycler as follows: 45 minutes to 1 hour at 37–55°C, 5–10 minutes at 95°C, then 40 cycles of: 94–95°C for 1 minute, 55°C for 1–1.5 minutes, 70–72°C for 2–2.5 minutes, followed by a final extension step of 7–8 minutes at 70–72°C. Analysis of the PCR products is carried out by agarose gel electrophoresis. AHS-positive samples will resolve in a 1179 base-pair band that can be used as template in the sequencing reaction, using the PCR primers independently to obtain the nucleotide sequence of viral segment 7.

1.2.3. Real-time RT-PCR procedure (Agüero *et al.,* 2008)

This group-specific real-time RT-PCR has been employed with very good results by the participating national reference laboratories of the EU Member States in annual proficiency tests for the period 2009–2015. Moreover, in an international ring trial organised in 2015 under the auspices of the OIE Reference Laboratory network, it was found to be one among other top-ranking protocols.

a) Protocol

The assay targets AHSV segment 7 (VP7) and is described in Agüero *et al.* (2008). It is able to detect all known AHSV types and strains currently circulating.

i) RNA extraction from blood and tissue samples

Commercial kits are widely available; the RNA extraction step can be performed according to the procedures specified in each kit.

ii) Several one-step real-time RT-PCR kits are commercially available that can be used depending on local or case-specific requirements, kits used and equipment available. Some basic steps as described by Agüero *et al.* (2008) are given below. (For primers and probe sequences see Table 2).

iii) Primer stock concentration is diluted to a working concentration of 8 µM whereas probe is diluted to a working concentration 50 µM.

iv) 2.5 µl of each primer working stock 8 µM (final concentration 1 µM) is added to each well of the PCR plate (or tube or strip) that will contain RNA samples, positive or negative controls. The plate is held on ice.

v) 2 µl of RNA samples, including test and positive and negative controls, is added to each well.

vi) Samples are subjected to heat denaturation at 95°C for 5 minutes, followed by rapid cooling on ice for further 5 minutes.

vii) An appropriate volume of real-time one-step RT-PCR master mix for the number of samples to be tested is prepared following the manufacturer's instructions. Probe

should be included in a final concentration of 0.25 µM (0.1 µl of probe working stock, 50 µM per sample).

viii) 13 µl of master mix is distributed in each well on the PCR plate containing the denatured primers and RNA.

ix) The plate is placed in a real-time thermal cycler programmed with the following profile:
48°C × 25 minutes
95°C × 10 minutes
40 cycles: 95°C × 15 seconds, 55°C × 35 seconds, 72°C × 30 seconds

If reagents and thermal cycler allowing fast reactions are employed then the following program can be used:
48°C × 25 minutes
95°C × 10 minutes
40 cycles: 97°C × 2 seconds, 55°C × 30 seconds
Fluorescence data are acquired at the end of the 55°C step.

Note: times and temperatures may vary and should be optimised for the reagents or kit used.

b) Interpretation of the results

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 to 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.

c) Diagnostic characteristics

i) Cut-off determination

The positive cut-off for the test method is less than 35 PCR cycles (Ct≤35).

The negative cut-off for the test method is 40 PCR cycles.

Test results between the positive and negative cut-offs (35 and 40 PCR cycles) are considered inconclusive (35 ≤ Ct ≤ 40).

ii) Diagnostic sensitivity and specificity

The diagnostic specificity (DSp) and diagnostic sensitivity (DSe) were calculated according to the procedure detailed in Chapter 1.1.6 *Principles and methods of validation of diagnostic assays for infectious diseases*.

In total 186 known negatives and 132 known positive samples were analysed to estimate DSe and DSp of the Agüero AHS real-time RT-PCR method, which is higher than the minimum number required (73) for an estimated DSe and DSp of 95% allowing a 5% error. DSe and DSp were 97% and 100%, respectively. It can thus be concluded that the number of known status samples used to calculate these diagnostic parameters was sufficient to comply with OIE requirements.

d) Reproducibility

Reproducibility of the Agüero real-time RT-PCR method in the international ring trial cited above was at least 93.55%, correctly identifying all positive and negative samples included in the panel. All laboratories detected dilutions of positive samples to at least 10^{-5}. Similar results have been reported in other proficiency test programmes.

Inactivated virus of serotypes 1–9 reference strains can be obtained from the OIE Reference Laboratory in Spain in order to set up the RT-PCR detection method.

1.2.4. Real-time RT-PCR procedure (Guthrie *et al.,* 2013)

a) **Protocol**

The test method presented here is adapted from Guthrie *et al.* (2013) and is capable of detecting all known AHSV types and strains currently circulating. The assay targets AHSV segment 7 (VP7). The procedure given may require modification to accommodate individual laboratory or different RT-PCR kit requirements.

i) RNA extraction from blood and tissue samples

Commercial kits are widely available; the RNA extraction step can be performed according to the procedures specified in each kit.

ii) Kits for the one-step real-time RT-PCR are available commercially. Below are some basic steps as described by Guthrie *et al.* (2013), which can be modified depending on local or case-specific requirements, kits used and equipment available.

iii) Primer and probe mix stock solutions are made up in a 25× concentration at 5 µM for the forward and reverse primers and 3 µM for the probe.

iv) 5 µl of RNA samples, including test and positive and negative controls, are added to appropriate wells of the PCR plate (or tube or strip).

v) Samples are subjected to heat denaturation at 95°C for 2 minutes, and held on ice for at least 3 minutes.

vi) An appropriate volume of real-time one-step RT-PCR master mix for the number of samples to be tested is prepared, following the manufacturer's instructions. 1 µl of 25× primer probe mix stock solution (from step iii above) is included in the master mix to give a final concentration in each well of 200 nM for each primer and 120 nM of the probe.

vii) 20 µl of master mix is distributed in each well on the PCR plate containing the denatured RNA.

viii) The plate is placed in a real-time thermal cycler programmed for reverse transcription and cDNA amplification or fluorescence detection as suggested by the manufacturers.

The following thermal profile is an example:
48°C × 10 minutes
95°C × 10 minutes
40 cycles: 95°C × 15 seconds, 60°C × 45 seconds

Note: times and temperatures may vary and should be optimised for the reagents or kit used.

b) **Interpretation of the results**

Samples are classified as "AHSV positive" if the normalised fluorescence for the AHSV real-time RT-PCR assay exceeds a 0.1 threshold within 36 PCR cycles in all replicates of a sample.

Samples are classified as "AHSV Inconclusive" if the normalised fluorescence for the AHSV real-time RT-PCR assay exceeds a 0.1 threshold between 36 and 40 PCR cycles in any replicate of a sample.

Samples are classified as "AHSV negative" if the normalised fluorescence for the AHSV assay did not exceed a 0.1 threshold within 40 PCR cycles in all replicates of a sample and if the normalised fluorescence for the internal positive control assay exceeded a 0.1 threshold within 33 PCR cycles.

Samples are classified as "no AHSV nucleic acid detected" if the normalised fluorescence for the AHSV assay did not exceed a 0.1 threshold within 40 PCR cycles in all replicates of a sample and if the normalised fluorescence for the internal positive control assay did not exceed a 0.1 threshold within 33 PCR cycles.

c) Diagnostic characteristics

i) Cut-off determination

The positive cut-off for the test method is less than 36 PCR cycles.

The negative cut-off for the test method is 40 PCR cycles.

Test results between the positive and negative cut-offs (36 and 40 PCR cycles) are considered inconclusive.

ii) Diagnostic sensitivity and specificity

The DSe and DSp of the AHSV real-time RT-PCR for detection of AHSV nucleic acid in whole blood samples were estimated by comparison with virus isolation using a two-test two-population Bayesian latent class model that allowed for conditional dependence (correlation) among test results. A total of 503 equine blood samples collected from individual horses with pyrexia and one or more clinical signs typical of AHS were used to represent AHS suspect cases. Blood samples were also collected from two separate healthy populations of horses (503 and 98 horses each, respectively) that were not vaccinated against AHS and that were highly unlikely to have been exposed to natural infection with AHSV; these samples were used to represent AHS negative cases.

The median diagnostic specificity of the test method exceeded 99.9%.

The median diagnostic sensitivity of the test method exceeded 97.8%.

d) Reproducibility

In the international ring trial cited above the Guthrie FRET probe real-time RT-PCR method demonstrated sensitivity in excess of 88.1%, a specificity of 100%, correctly identifying all positive and negative samples included in the panel. All laboratories detected dilutions of positive samples to at least 10^{-5}.

Table 2. *Comparison of the real-time RT-PCR methods of Agüero* et al. *(2008) and Guthrie* et al. *(2013)*

	Agüero *et al.,* 2008	Guthrie *et al.,* 2013
Target	Group specific (VP7)	Group specific (VP7)
Primers (5'-3')	CCA-GTA-GGC-CAG-ATC-AAC-AG	AGA-GCT-CTT-GTG-CTA-GCA-GCC-T
	CTA-ATG-AAA-GCG-GTG-ACC-GT	GAA-CCG-ACG-CGA-CAC-TAA-TGA
Probe (5'-3')	FAM-GCT-AGC-AGC-CTA-CCA-CTA-MGB	FAM-TGC-ACG-GTC-ACC-GCT-MGB
Annealing temperature	55°C	60°C
Number of amplification cycles	40	40
Analytical sensitivity (LOD)	Dilution 10^{-5} of a viral suspension of AHSV-4 reference strain with a titre of $10^{6.3}$ TCID$_{50}$/ml, which corresponded to a Ct of 34.3±0.5 ($10^{1.3}$ TCID$_{50}$/ml)	Dilution of 3.02×10^{-6} of a AHSV positive blood samples, with corresponding Ct of 35.71
Diagnostic specificity	100%	99.9%.
Diagnostic sensitivity	97%	97.8%.

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; Sailleau *et al.,* 2000), and real-time RT-PCR using hybridisation probes (Koekemoer, 2008) targeting AHSV Seg-2 for identification and differentiation of AHSV genotypes, provides a rapid typing method for AHSV in tissue samples and blood. These methods can be used to very significantly increase the speed and reliability of detection and identification (compared with VN tests) of the nine serotypes of AHSV. Type-specific real-time RT-PCR assays based on the use of Taqman-MGB probes have been developed recently by Bachanek-Bankowska *et al.*, 2014, Weyer *et al.,* 2015).

However, the genetic variation that may appear over time in the AHSV genome, in particular 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 are able to 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 (Hamblin *et al.*, 1990; Laviada *et al.*, 1992; Maree & Paweska, 2005; Wade Evans *et al.,* 1993) have proved to be good methods for the detection of anti-AHSV group-reactive antibodies, especially for large-scale investigations (Rubio *et al.*, 1998). Both of these tests have been recognised by the European Commission (2002). 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 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 (European Commission, 2002).

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.

2.1.1. Test procedure

i) *Solid phase:* coat 96 well ELISA plates with 50–100 ng of recombinant AHSV-4 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. This MAb has been diluted previously 1/5000–1/15000 in a 1/1 solution of StabiliZyme Select® Stabilizer (SurModics. Reference: SZ03) in distilled water. 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, i.e. 1 ml of ABTS (2,2'-azino-di-[3-ethyl-benzothiazoline]-6-sulphonic acid), 5 mg/ml + 9 ml substrate buffer

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

$$BP = \frac{Abs(control^-) - Abs(sample)}{Abs(control^-) - Abs(control^+)} \times 100$$

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[2] 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.

2.2.1. Test 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 (or 620 nm).

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.

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2   The antigen can be provided on request by the Centro de Investigación en Sanidad Animal (CISA), Spain. The delivery time is 4–6 months.

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[3] 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 1/180.

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.

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3 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.

2.4. Virus neutralisation (VN)

Serotype-specific antibody can be detected using the VN test (House *et al.*, 1990). The VN test 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 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 in the assay that cannot neutralise 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 in the assay that can neutralise 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 fuschsin.

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 (Erasmus, 1976; Sanchez-Vizcaíno, 2004). Polyvalent vaccines are commercially available.

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 available at the present time. 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.

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 1×10^6 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 1×10^6 PFU/ml.

Finally, two multivalent vaccines are constituted by mixing equal volumes of serotypes 1, 3, 4 and 2, 6, 7, 8 respectively. 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 1×10^3 PFU/dose. The infectivity titre of the final product is assayed by plaque titration in Vero cell cultures and should contain at least 1×10^5 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 authorisation

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 authorisation 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 subunit vaccines have been described (Section C.1.1 *Rationale and intended use of the product*).

4.2. Special requirements for biotechnological vaccines, if any

None.

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

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2017