

EQUIDAE

AFRICAN HORSE SICKNESS

SUMMARY

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. Only AHS serotype 9 and 4 have been found in 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. Over the past few years, 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 important to perform virus isolation and serotyping whenever outbreaks occur outside the enzootic regions.*

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) or African green monkey kidney (Vero), intravenously in embryonated eggs, and intracerebrally in newborn mice. Several enzyme-linked immunosorbent assays (ELISAs) for the rapid detection of AHSV antigen in 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 method. Virus isolates can be serotyped by a type-specific serological test such as virus neutralisation (VN) and by reverse-transcription polymerase chain reaction and 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. Other tests that have been described are immunodiffusion and haemagglutination inhibition.*

Requirements for vaccines and diagnostic biologicals: *Attenuated (monovalent and polyvalent) live vaccines for use in horses, mules and donkeys, are currently commercially available. A monovalent inactivated vaccine has been produced commercially, but is no longer available. New vaccines, including a subunit vaccine, have been evaluated experimentally.*

A. INTRODUCTION

African horse sickness (AHS) (*Peste equina africana*, *Peste equine*) is an infectious, noncontagious arthropod-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. 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, which encode seven structural proteins (VP1-7), most of which have been completely sequenced for AHSV serotypes 4, 6 and 9 (29, 36, 39) and four nonstructural proteins (NS1, NS2, NS3, NS3A) (11, 19). 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 (35), the most variable being the major outer capsid protein, VP2. This protein, VP2, is also the principal responsible for AHSV serotypes and, together with VP5, for virus neutralisation activity (26). Nine antigenically distinct serotypes of AHSV have been identified by virus neutralisation but 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 have been observed.

AHS is enzootic in sub-Saharan Africa, although occasional outbreaks have occurred in northern Africa (1965, 1989–1990), the Middle East (1959–1961), and in Europe (Spain, 1966, 1987–1990 and Portugal, 1989).

The disease has both a seasonal (late summer/autumn) and a cyclical incidence with major epizootics in southern Africa during warm-phase events (1). Mortality due to AHS is related to the species of equidae affected and to the strain or serotype of the virus. At least two field vectors are involved: *Culicoides imicola* and *C. bolitinos*. Among the 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) (4).

B. DIAGNOSTICS TECHNIQUES

Although some clinical signs and lesions are characteristic, for example the supraorbital swelling that is often present in horses with subacute AHS, these clinical signs combined with an appropriate history and epidemiological information may be sufficient for a tentative diagnosis. However, other signs and lesions are less specific for AHS, and other diseases such as equine encephalosis, equine infectious anaemia, equine morbillivirus pneumonia, equine viral arteritis, babesiosis and purpura haemorrhagica may be confused with one or other forms of AHS and should be excluded. These are the reasons why a laboratory diagnosis is essential to establish a correct and confirmatory diagnosis. This is the reason that laboratory confirmation is essential.

1. AHS clinical forms

There are four classical clinical forms of AHS: pulmonary, cardiac, mixed, and horse sickness fever (7).

The peracute or pulmonary form, which has a short incubation period (3–5 days), is characterised by severe dyspnoea and progressive respiratory involvement. An acute febrile reaction, lasting 1–2 days and reaching a maximum of approximately 40–41°C, may be the only sign. This is followed by various degrees of respiratory distress – respiratory rate may increase to 60 or even 75 breaths/minute. The animal may be observed to stand with its forelegs spread apart, its head extended and its nostrils fully dilated. Profuse sweating is common and spasmodic coughing may be observed terminally, with frothy fluid exuding from the nostrils. Death usually occurs within a few hours after the first clinical signs are observed, the animal having literally drowned in its own serous fluid. The pulmonary form is usually observed in completely susceptible animals, animals infected with a highly virulent strain of virus, or animals that are worked during the febrile stage of the disease. Recovery from this form is very rare, occurring in <5% of cases. This is also the form usually seen in dogs.

The incubation period of the subacute, oedematous or cardiac form varies from about 7 to 14 days, and the onset of clinical disease is marked by a febrile reaction (39–41°C) that lasts for 3–6 days. Shortly before the decline of the fever, characteristic oedematous swellings may appear. These initially involve the temporal or supraorbital fossae and the eyelids, and later extend to the lips, cheeks, tongue, intermandibular space and laryngeal region. Subcutaneous oedema sometimes extends a variable distance down the neck towards the chest and, in severe cases, may involve the chest and shoulders, but generally not the lower limbs. Terminally, petechial haemorrhages may be observed in the conjunctivae and on the ventral surface of the tongue. The animal finally becomes restless and may show signs of colic before death from cardiac failure. Difficulty in swallowing due to paralysis of the oesophagus is also seen. The mortality rate is about 50% and death usually occurs within 4–8 days after the onset of the febrile reaction. In recovering cases, swelling gradually subsides within a period of 3–8 days. This clinical form of AHS is usually associated with infection by virus strains of low virulence or is

encountered in immune animals infected by heterologous virus strains, or may be a function of biological variation in the infected animal.

The acute or mixed form represents a mixture of the pulmonary and cardiac forms and is often the most common form of AHS in horses and mules. The mortality rate is around 70% within 3–6 days after the onset of the febrile reaction. This form of the disease may manifest itself in the following ways:

- Initial pulmonary signs of a relatively mild degree are followed by marked oedematous swellings of the head and neck, with death resulting from heart failure.
- Oedematous swelling, typical of the subacute form, is followed by the sudden onset of dyspnoea and other clinical signs typical of the peracute pulmonary form.

Horse sickness fever is the mildest form and is frequently overlooked in natural outbreaks. The incubation period varies from 5 to 14 days, and is followed by a febrile reaction (39–40°C) of the remittent type, with morning remissions and afternoon exacerbations, lasting for 5–8 days. Apart from the febrile reaction, other clinical signs are rare. The conjunctivae may be slightly congested, the pulse rate may be increased, and a certain degree of anorexia, depression and oedema of supraorbital fossae may be present. There is no mortality. This form of the disease is usually observed in partially immune animals or in resistant species, such as the donkey and zebra.

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, certain neurotropic vaccine strains that may cause encephalitis and meningitis in humans following transnasal infections have been described (28). Experimental and natural transmission of AHS to dogs has been reported through ingestion of infected horse meat (3). However, there is only limited and unsubstantiated evidence that dogs become infected by insect bites (34).

2. Isolation and identification of the agent

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 a new reverse-transcription (RT) PCR for discrimination of the nine AHSV serotypes (31), or cell culture and inoculation of newborn mice. 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 should be performed as early in the outbreak as possible so that the correct vaccine can be selected. Subsequently, the ELISA may be very useful in laboratory diagnosis.

At present, there are no international standards for viruses or diagnostic reagents, and there is no standard methodology for the determination of AHSV. However, a viral panel has been evaluated, and comparative studies between different ELISAs for AHSV antigen determination have been carried out in different laboratories. The results have demonstrated a high level of correlation for antigen detection (30) using the indirect sandwich ELISAs for antigen studies (13, 18).

A very important aspect of the diagnosis is the selection of samples and their transportation to the laboratory.

a) Samples for 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 storage.

b) 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 observed 7–10 days after infected insect cells are passed onto mammalian cells.

c) Newborn mice

This method of isolation of AHSV involves the intracerebral inoculation of two families of 1–3-day-old mice. In positive cases, animals develop nervous signs between 3 and 15 days post-inoculation. The brains from sick animals may be collected, homogenised and re-inoculated intracerebrally into at least six 1–3-day-old mice. This second passage should present a shortened incubation period (2–5 days) and 100% infectivity. Virus may be typed directly from mouse brain by conventional neutralisation (VN) or by RNA extraction and sequencing.

d) Sandwich enzyme-linked immunosorbent assay

At least two serogroup-specific sandwich ELISAs have been developed and field tested for detection of AHSV antigen from both field samples and laboratory-infected tissue cultures (13, 18).

One technique (13) uses PABs to the AHSV and the other (18) uses MAbs against one of the major proteins that is more conserved among serotypes – protein VP7. Both methods have been demonstrated to be adequate for the diagnosis of AHS, due to their high sensitivity and specificity and the availability of results in only 2–4 hours (30). The use of a chicken IgY in a double-antibody sandwich ELISA for detecting all AHSV serotypes has also been described (6).

Reagents for the ELISA may be obtained from the OIE Reference Laboratories for African horse sickness (see Table given in Part 3 of this *Manual*).

• **The following is an example of a monoclonal antibody enzyme-linked immunosorbent assay**

- i) *Solid phase*: Coat ELISA plates (e.g. high adsorption capacity Nunc Maxisorb) with a mixture of MAb 5G5 and 3D2 diluted in PBS, pH 7.2 (10 µg/ml each). Incubate overnight at 4°C.
- ii) Wash the plates five times with distilled water containing 0.05% (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 + 1% bovine serum albumin (BSA), pH 7.2, 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*: Add the samples to be tested (twofold dilutions starting with undiluted spleen homogenates, or AHSV cell culture supernatant) diluted in PBS + 1% BSA, pH 7.2, 100 µl/well. Incubate for 1 hour at 37°C. (Spleen homogenate: homogenise approximately 2 cm³ [1 g] of spleen with 3 ml of MEM [minimal essential medium] culture medium. Centrifuge at 600 **g** for 10 minutes and save the supernatant.)
- vi) Wash the plates as described in step ii.
- vii) *Conjugate*: Dispense 100 µl/well of biotin-labelled 5G5 MAb diluted 1/500 in PBS + 1% BSA, pH 7.2. Incubate for 1 hour at 37°C. Wash the plates as described in step ii. Add 100 µl/well of avidin/oxidase at optimal dilution in PBS + 1% BSA. Incubate for 45 minutes at room temperature.
- viii) Wash the plates as described in step ii.
- ix) *Substrate*: Add 200 µl/well of substrate solution (10 ml of 80.6 mM DMAB [dimethyl amino-benzaldehyde] + 10 ml of 1.56 mM MBTH [3-methyl-2-benzo-thiazolinone hydrazone hydrochloride] + 5 µl H₂O₂). Colour development is stopped by adding 50 µl of 3 N H₂SO₄ after approximately 5–10 minutes (before the negative control begins to be coloured).
- x) Read the plates at 600 nm (or 620 nm).
- xi) *Interpretation of the results*: Calculate the cut-off value as follows: $C \pm 0.06 = \text{cut-off}$ (where C is the absorbance value obtained with the negative control). Test samples giving absorbance values lower than the cut-off are regarded as negative. Test samples giving absorbance values greater than the cut-off + 0.20 are regarded as positive. Test samples giving intermediate absorbance values are doubtful and a second technique must be employed to confirm the result.

e) Polymerase chain reaction

An RT-PCR assay for the specific detection of AHSV genome has been developed. This assay (method 1) can be used to detect viral RNA in blood collected in EDTA (ethylene diamine tetra-acetic acid), homogenised equid or mouse tissue or cell culture fluids. Primers correspond to the 5' end (nucleotides 1–21) and 3' end (nucleotides 1160–1179) of RNA segment 7 (4, 22, 33, 40), amplifying the complete segment 7 of the genome. More recently, a new conventional technique (method 2) and a real-time RT-PCR (method

3) technique have been developed using primers from a highly conserved region of the same viral genome segment 7, to achieve better sensitivity and rapidity in the diagnosis of AHS (10). The three RT-PCR procedures can detect the nine virus serotypes and are described below.

- **Test procedure - method 1 (4)**

Extraction of nucleic acids from spleen samples is carried out as follows: 1 g of tissue sample is homogenised in 1 ml of denaturing solution (4 M guanidium thiocyanate, 25 mM sodium citrate, 0.1 M 2-mercaptoethanol, 0.5% sarcosyl). After centrifugation, 1 µg of yeast RNA, 0.1 ml of 2 M sodium acetate pH 4, 1 ml of phenol and 0.2 ml of chloroform/isoamyl alcohol mixture (49/1) are added to the supernatant. The suspension is vigorously shaken and cooled on ice for 15 minutes. After centrifugation, the RNA present in the aqueous phase is phenol extracted, ethanol precipitated and resuspended in sterile water. The methods for cDNA synthesis and PCR amplification are performed using, in all cases, 37°C as renaturing temperature. 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. The PCR procedure itself involves 40 cycles (94°C for 1 minute, 55°C for 1.5 minutes, 72°C for 2.5 minutes and 70°C for 7 minutes) and then the PCR tubes are kept at 4°C. Analysis of the PCR products is carried out by electrophoresis in 1.2% (w/v) agarose gels containing ethidium bromide. AHS-positive samples will resolve in a 1179 base-pair band.

- **Test procedure - method 2 (10)**

Viral double-stranded RNA extraction procedure can be achieved using the commercial High Pure Viral Nucleic Acid Kit (Roche Diagnostics), which is described below. A number of other RNA extraction kits are commercially available for the preparation of template suitable for RT-PCR depending on the sample submitted for analysis and may be appropriate for use. Different samples can be used in this procedure such as cell culture supernatants, EDTA/blood, serum or tissue homogenates.

The High Pure Viral Nucleic Acid Kit (Roche Diagnostics) includes the following reagents: Binding Buffer, Poly (A) carrier RNA, Proteinase K, Inhibitor Removal Buffer, Wash Buffer, and High Pure Filter Tubes and collection tubes.

For organ and tissue samples, first prepare a 1/10 homogenate of the material in PBS, then centrifuge to clarify at 12,000 *g* for 5 minutes. Extract RNA from the resultant supernatant fluid. Sometimes it is recommended to process a 1/10 dilution of the supernatant in parallel.

Extraction for control samples: 1/10 tissue homogenates (same tissue as samples to be analysed): (a) a negative control: use 200 µl of a homogenate of AHSV-negative tissue; (b) a positive control: use 200 µl of a homogenate of AHSV-positive tissue. Process both controls together with the test samples.

- Pipette 200 µl of sample into a 1.5 ml microcentrifuge tube.
- Add 200 µl of binding buffer supplemented with poly(A) and 50 µl of proteinase K. Mix immediately. Incubate for 10 minutes at 72°C.
- Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.
- Mix samples with 100 µl of binding buffer. Briefly centrifuge to remove drops from the inside of the lid.
- Place the High Pure filter tube in a collection tube and pipette the sample in the upper reservoir. Centrifuge for 1 minute at 8000 rpm (with blood samples, repeat the centrifugation step if sample remains in the filter tube).
- Discard the flowthrough and the collection tube, and place the filter tube into a clean collection tube.
- Add 500 µl of Inhibitor Removal Buffer to the upper reservoir and centrifuge for 1 minute at 8000 rpm.
- Discard the flowthrough and the collection tube, and place the filter tube into a clean collection tube.
- Add 450 µl of wash buffer to the upper reservoir and centrifuge for 1 minute at 8000 rpm.
- Discard the flowthrough and the collection tube, and repeat the washing step.
- Discard the collection tube and place the filter tube into a clean collection tube. Centrifuge for 10 seconds at 13,000 rpm to remove residual wash buffer.
- Discard the collection tube and place the filter tube in a clean 1.5 ml microcentrifuge tube.
- For the RNA elution, add 50 µl of prewarmed (70°C) RNase free sterile water to the upper reservoir. Centrifuge for 1 minute at 8000 rpm.

xiv) Use immediately or store at -20°C for future use.

- **Stock solutions for RT-PCR**

- Nuclease-free sterile water.
- *One-step RT-PCR kit* is commercially available from *Qiagen*, that contains RT and PCR enzymes, PCR nucleotide mix, PCR buffer with magnesium chloride, and Q solution.
- RNase inhibitor enzyme is commercially available from several suppliers.
- Primers at a concentration of 20 pmol/ μl : Primer 1 sequence 5'-GGC-TCC-AAC-ACT-CAC-AAG-ATG-T-3' (forward primer); primer 2 sequence 5'-GGC-GGA-TTA-ATA-GGC-TGC-ATA-3' (reverse primer).
- *10x Loading buffer*: 0.2% xylene cyanol, 0.2% bromophenol blue, 30% glycerol.
- *TAE buffer (50x) for agarose gel*: Tris base (242 g); glacial acetic acid (57.1 ml); 0.5 M EDTA, pH 8.0 (100 ml); distilled water (to 1 litre).
- *Marker DNA*: 100 base-pair ladder is commercially available.

- **RT-PCR amplification assay**

- Denaturation of the viral double-stranded RNA is recommended to be performed prior to the RT-PCR step. Prepare the following mixture for each sample: primer 1, 20 pmol/ μl (0.5 μl), primer 2, 20 pmol/ μl (0.5 μl), 7 μl of nuclease-free sterile water, and 2 μl of extracted sample template.
- Place the tubes in a thermal cycler or heating block, and incubate at 95°C for 5 minutes. Briefly centrifuge the tubes to remove drops from the inside of the lid, and immediately, place the tubes on ice.
- In a sterile 1.5 ml microcentrifuge tube prepare the RT-PCR reaction mixture described below for each sample. Prepare the reaction mixture in bulk for the number of samples to be assayed allowing for one extra sample.
- Nuclease-free sterile water (3.25 μl), PCR Buffer 5x with magnesium chloride (5 μl), dNTP mix 10 mM (0.5 μl), Q solution 5x (5 μl), RNase inhibitor 20 U/ μl (0.25 μl), enzymes mix (1 μl).
- Add 15 μl of the RT-PCR reaction mix to each PCR tube containing the denatured RNA template.
- Include a positive reaction control (2 μl of AHSV RNA) and a negative reaction control (2 μl of distilled water) for each RT-PCR run.
- Place all the tubes in an automated thermal cycler and run the following programme:
 - One cycle at 55°C for 30 minutes.
 - One cycle at 95°C for 15 minutes.
 - 40 cycles at 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds.
 - One cycle at 72°C for 7 minutes.
 - Hold at 4°C .
- At the end of the programme, remove PCR tubes and add 2.5 μl of 10x loading buffer to each tube.
- Load all the samples in a 3% agarose gel in TAE buffer containing ethidium bromide at a final concentration of 0.5 $\mu\text{g}/\text{ml}$. Add marker DNA to one lane on each side of the gel.
- Run the gel at a constant voltage of 150–200 volts for about 30 minutes.
- Reading the results*: Examine the gel over a UV light source. In a positive sample, a discrete band will be present that should co-migrate with the PCR product of the positive control. Calculate the size of the PCR products in the test samples and the positive control by reference to the standard markers. The PCR product of the positive control has a size of 102 base pairs. No bands should be seen in the negative control.

- **Test procedure - method 3: real-time RT-PCR (10)**

Follow the sample preparation and viral RNA extraction procedure as described for the conventional RT-PCR method 2 described above.

- **Stock solutions for RT-PCR**

- Nuclease-free sterile water.

- *QuantiTect probe RT-PCR kit* is commercially available from *Qiagen*, which contains RT enzymes (RT mix) and PCR enzymes, PCR nucleotide mix, PCR buffer with magnesium chloride (master mix).
 - RNase inhibitor enzyme is commercially available from several suppliers.
 - Use the same primers than in the conventional RT-PCR method 2 described above. Primers at a concentration of 20 pmol/μl: Primer 1 sequence 5'-GGC-TCC-AAC-ACT-CAC-AAG-ATG-T-3' (forward primer); primer 2 sequence 5'-GGC-GGA-TTA-ATA-GGC-TGC-ATA-3' (reverse primer).
 - TaqMan-MGB[®] probe at a concentration of 10 pmol/μl: (5'-[6-carboxy-fluorescein (FAM)]-TGG-CAC-GCC-TTA-CGC-GC-[minor groove binder molecule (MGB)]-3').
- **RT-PCR amplification assay**
 - i) Denaturation of the viral double-stranded RNA is recommended to be performed prior to the RT-PCR step. Prepare the following mixture for each sample: primer 1 20 pmol/μl (0.75 μl), primer 2 20 pmol/μl (0.75 μl), 6.5 μl of nuclease-free sterile water, and 2 μl of extracted sample template.
 - ii) Place the tubes in a thermal cycler or heating block, and incubate at 95°C for 5 minutes. Briefly centrifuge the tubes to remove drops from the inside of the lid, and immediately, place the tubes on ice.
 - iii) In a sterile 1.5 ml microcentrifuge tube, prepare the RT-PCR reaction mixture described below for each sample. Prepare the reaction mixture in bulk for the number of samples to be assayed allowing for one extra sample.
 - iv) Nuclease-free sterile water (1.375 μl), TaqMan-MGB[®] probe 10 pmol/μl (0.625 μl) Master mix 2x (12.5 μl), RNase inhibitor 20 U/μl (0.25 μl), RT mix (0.25 μl).
 - v) Add 15 μl of the RT-PCR reaction mix to each optical PCR tube or to each well of an optical reaction plate, containing the denaturated RNA template.
 - vi) Include a positive reaction control (2 μl of AHSV RNA) and a negative reaction control (2 μl of distilled water) for each RT-PCR run.
 - vii) Place all the tubes in an automated real-time thermal cycler and run the following program:
 - One cycle at 55°C for 30 minutes.
 - One cycle at 95°C for 15 minutes.
 - 45 cycles at 94°C for 15 seconds, 60°C for 1 minute. Make the fluorescence measurement at the end of each cycle in the appropriate channel.
 - viii) *Reading the results:* Assign a threshold cycle (Ct) value to each PCR reaction from a scan of all amplification plots (a plot of the fluorescence signal versus cycle number). Negative test samples, uninfected negative or extraction blank controls should have a Ct value >45.0. Positive test samples and controls should have a Ct value <45.0 (strongly positive samples have a Ct value <30.0).

- **AHSV serotyping**

Until recently VN test has been the method of choice for serotyping as well as the 'gold' standard test for identifying AHSV's isolated from the field using type specific antisera (37). This technique takes five or more days before results are obtained. The recent development of a type-specific RT-PCR for identification and differentiation of the nine AHSV serotypes provided a method of confirming the identity of AHSV in tissue samples within a few hours (27, 31). Nine pairs of primers were designed in the virus genome segment 2 for each specific serotype. The results obtained show a perfect agreement between the RT-PCR and the VN test.

Typing of nine AHS serotypes has also been carried out with probes developed from a set of cloned full-length VP2 genes and can be an alternative to amplification of genome segment 2 (17).

3. Serological tests

OIE International Reference sera are available (consult the OIE web site [www.oie.int] for the address). These sera were developed to standardise the ELISA, which is an OIE prescribed test. In addition, a panel of reference antiserum has been evaluated and comparative studies between different ELISAs using MAb and PAb, and involving several laboratories have been carried out. The results have demonstrated a high level of correlation using indirect or competitive ELISAs for antibody detection (12, 20, 30). More recently, a panel of sera has been generated and is now being used for the annual quality assurance of three antibody detection ELISAs as used by the national laboratories in Europe (9, 12, 20).

Indirect and competitive ELISAs using either soluble AHSV antigen or a recombinant protein VP7 (12, 20) have proved to be good methods for the detection of anti-AHSV group-reactive antibodies, especially for large-scale investigations (30). Both of these tests have been recognised by the European Commission (9). The competitive ELISA can also be used for testing wildlife as species-specific anti-globulin is not required with this method. An immunoblotting test has been adapted for anti-AHS antibody determination (20). It is especially suitable for small numbers of sera. An indirect ELISA is also available that uses the AHSV serotype 4 nonstructural protein NS3 as antigen. The assay can be used to differentiate between animals infected or vaccinated with the live vaccine from those vaccinated with an inactivated vaccine made with purified virions (21). However, it should be noted that no such inactivated vaccine is at present on the market. The complement fixation (CF) test has been widely used, but some sera are anti-complementary, particularly donkey and zebra.

a) Indirect enzyme-linked immunosorbent assay (a prescribed test for international trade)

The recombinant VP7 protein has been used as antigen for AHSV antibody determination with a high degree of sensitivity and specificity (20, 38). Other advantages of this antigen are its stability and its lack of infectivity (23).

• **Test procedure**

- i) *Solid phase:* Coat ELISA plates (e.g. high adsorption capacity Nunc Maxisorb) 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, 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) *Substrate:* Add 200 µl/well of substrate solution (10 ml DMAB + 10 ml of MBTH + 5 µl H₂O₂). Colour development is stopped by adding 50 µl of 3 N H₂SO₄ after approximately 5–10 minutes (before the negative control begins to be coloured). Other substrates such as ABTS (2,2'-azino-di-[3-ethyl-benzothiazoline]-6-sulphonic acid), TMB (tetramethyl benzidine), or OPD (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 0.6 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.

b) Immunoblotting

The binding of antibodies to viral proteins separated by electrophoresis and transferred to nitrocellulose paper has been used for the determination of anti-AHSV antibodies (20).

• **Test procedure**

Semipurified proteins of AHSV are separated by SDS/PAGE (sodium dodecyl sulphate/polyacrylamide gel electrophoresis) in 15% (w/v) acrylamide-N,N'-diallyltartar-diamide (DATD) gels. Separated proteins are transferred to a nitrocellulose membrane filter at a constant current of 280 mA for 6 hours at 4°C. Immunoblotting of a nitrocellulose membrane cut into strips is carried out using the sera at 1/20 dilution, peroxidase-conjugated rabbit-anti-horse immunoglobulin at 1/500 dilution and an incubation time of 1 hour at 37°C. The bands recognised by the sera are developed by the 4-chloro-naphthol technique.

Interpretation of results: The comparison between the band pattern of a positive control and a negative control serum permits the identification of the specific viral bands. The appearance of two or more of these specific bands in a problem serum allows it to be classified as positive anti-AHS serum.

c) NS3 enzyme-linked immunosorbent assay

An indirect ELISA to distinguish between infected horses and horses vaccinated with an inactivated purified AHSV serotype 4 vaccine, using a recombinant NS3 protein as antigen, has been described (21). The results obtained indicate that recombinant NS3 can differentiate between infected animals and those vaccinated animals vaccinated with an inactivated vaccine, implying that this recombinant could be an important diagnostic reagent that could allow the transportation of vaccinated horses. To ensure the reliability of results it is essential that the selected AHSV-inactivated vaccine be a purified exclude with certainty any trace of NS3 that, if present, would stimulate the production of anti-NS3 antibodies vaccine. This is to in vaccinated horses, thus mimicking the response to a natural infection. This type of ELISA would also be useful for distinguishing between infected horses and horses vaccinated with a subunit vaccine in which NS3 protein is not present. However, it should be noted that no inactivated vaccines against AHS are at present on the market.

d) Complement fixation (a prescribed test for international trade)

The CF test has been used extensively, but due to the anti-complementary effect of some sera, as well as the good results obtained with the ELISA, its use is decreasing. The CF test is frequently used for the demonstration of group-specific antibodies against AHSV. A sucrose/acetone mouse-brain extract is commonly used as antigen.

- **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.
- 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 solution¹ 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.

- **Test procedure**

- i) Sera, complement and antigen are reacted in 96-well round-bottom microtitre plates, or in tubes if the macro-technique is used, at 4°C for 18 hours.
- ii) Sensitised SRBCs (3%) are added to all wells on the microtitre plate.
- iii) The test plate is incubated for 30 minutes at 37°C.
- iv) Plates are then centrifuged at 200 **g**, and the wells are scored for the presence of haemolysis.
- v) The following controls are used: (a) serum and complement; (b) serum and SRBCs; (c) CF antigen and control antigen each with 4 CH₅₀ (50% complement haemolytic units), 2 CH₅₀, and 1 CH₅₀ of complement; (d) CF antigen and SRBCs; (e) control antigen and SRBCs; (f) complement dilutions of 4 CH₅₀, 2 CH₅₀, and 1 CH₅₀, and (g) SRBCs.
- vi) 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.
- vii) A titre of 1/10 or more is positive, under 1/10 is negative.

e) Virus neutralisation (VN)

¹ 20.5 g dextrose (114 mM), 7.9 g sodium citrate 2H₂O (27 mM), 4.2 g NaCl (71 mM), H₂O to 1 litre. Adjust to pH with 1 M citric acid.

Serotype-specific antibody can be detected using the VN test (14, 15). 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 (27).

- **VN Test procedure**

- i) Stock virus is diluted to yield 100 TCID₅₀ (50% tissue culture infective dose), with a range of 30–300 TCID₅₀, per 25 µl, and 25 µl is added to each of four microtitre wells containing 25 µl serum dilutions. For screening, a final serum dilution of 1/10 is used. Doubling dilutions are used for titrations.
- ii) Serum/virus mixtures are incubated for 60 minutes at 37°C 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% CO₂, 95% humidity for 4–5 days, until the back titration indicates that the stock virus contains 30–100 TCID₅₀.
- iv) The plates are then fixed and stained in a solution of 0.15% (w/v) crystal violet in 2% (v/v) glutaraldehyde and rinsed. Alternatively, they may be fixed with 70% ethanol and stained with 1% basic fuchsin.
- v) The 50% end-point titre of the serum is calculated by the Spearman–Kärber method and expressed as the negative log₁₀.

C. REQUIEREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Polyvalent or monovalent live attenuated AHS vaccines based on the selection in Vero cell culture of genetically stable macroplaques are commercially available from Onderstepoort Biological Products, Onderstepoort, South Africa (8). An inactivated monovalent (serotype 4) AHSV vaccine based on virus purification and inactivation with formalin has been produced commercially, but is not available at the present time (5, 16). Requirements for both attenuated and inactivated vaccines are summarised below.

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.

C1. Attenuated African horse sickness vaccine

1. Seed management

a) Characteristics of the seed

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.

b) Method of culture

The seed virus is grown in roller cultures of Vero cells.

c) Validation as a vaccine

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.

2. Method of manufacture

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 × 10⁶ plaque-forming units (PFU)/ml of infectious virus.

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. 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 quadrivalent vaccines are constituted by mixing equal volumes of serotypes 1, 3, 4, 5 and 2, 6, 7, 8 respectively. Subsequently, AHSV serotype 5 was withdrawn from this vaccine. 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.

4. Batch control

a) 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 products are given in Chapter 1.1.9.

b) 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.

c) 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.

d) Duration of immunity

Duration of immunity is not assessed with every batch of vaccine, but immunity is known to persist for at least 4 years. However, in the light of possible interference between the individual serotypes in each quadrivalent vaccine, annual revaccination is advocated in enzootic regions. Vaccination with monovalent vaccine stimulates a practically lifelong immunity.

e) Stability

In the lyophilised state, the vaccine is known to retain its potency for many years when stored at 4–8°C. However, an expiration date of 2 years is normally given.

C2. Inactivated African horse sickness vaccine

1. Seed management

a) Characteristics of the seed

The seed virus is the attenuated vaccine strain (AHSV serotype 4) used widely in the field in Africa and southern Europe (seed stocks are available from the OIE Reference Laboratory [Onderstepoort]). The virus was passaged ten times in the brains of newborn mice for attenuation, and then passaged a further ten times in roller tube cultures of BHK-21 cells. This material was plaque-purified three times in Vero cell cultures by selection of a large plaque (4–6 mm) at terminal dilutions. The final plaque material was passaged once in the brain of newborn mice and four times in Vero cell cultures. This material was lyophilised and constitutes the master seed virus.

b) Method of culture

The attenuated virus is propagated by passage in BHK-21 cells in roller bottles.

c) Validation as a vaccine

The seed virus must be shown to be free of contaminating viruses, bacteria and mycoplasmas by the appropriate techniques. The serotype identification is confirmed by a double-sandwich ELISA using MAbs.

2. Method of manufacture

Virus is harvested when characteristic CPE is fully developed. Cultivation thereafter is carried out in cell suspensions with serum-free Stoker's medium (MEM) at 37°C. Virus growth is checked by cell viability (CPE), and when optimum conditions are reached, the fermentor culture temperature is lowered to 4°C. The virus suspension is aseptically harvested.

The filtered suspension of attenuated virus is inactivated with formaldehyde at a final concentration of 1/1,000. The suspension is transferred to another tank and kept at 4°C under mild agitation for at least 10 days to ensure that all virus is inactivated. Inactivation controls are also performed.

After inactivation, the antigen suspension is concentrated by ultrafiltration, and then purified by selective precipitation by a complex of ethylene oxide and bivalent cations according to a patented process.

3. In-process control

Control of virus identity by double-sandwich ELISA, titration of virus before inactivation in Vero cell lines, sucrose gradient analysis to estimate the viral particle concentration, and sterility tests are performed.

4. Batch control

a) Sterility

Tests for sterility and freedom from contamination of biological products are given in Chapter 1.1.9.

b) Safety

AHSV-susceptible horses are inoculated intramuscularly or subcutaneously with a single or double dose of vaccine. Temperatures are recorded daily for 14 days, and horses are observed for signs of abnormality. Five guinea-pigs receive a horse vaccine dose by the intramuscular route. Sera collected at day 21 are tested for antibodies by VN.

c) Potency

The potency control is performed by challenge of vaccinated horses. Some animals are vaccinated with one dose, and others with two doses, of vaccine. The challenge is made by the intravenous route at 77 days after the first vaccine inoculation. Where two vaccinations are used, the second dose is given 21 days after the first dose.

Serum samples are collected on the day of vaccination and 21 days later, and are tested for neutralising antibodies and virus isolation.

d) Duration of immunity

No challenges have been performed on horses; only serological studies have been carried out. If the level of antibodies after two injections (recommended field protocol) and at 12 months post-vaccination is equivalent to the levels conferred by a single vaccination at 28 days post-vaccination, the horses are considered to be protected. With this standard, the duration of immunity is 1 year, starting 7–10 days after the first injection. One booster every year is sufficient to confer a protective level in the following years. The protocol for vaccination recommended by the manufacturer is two vaccinations (given 21 days apart) and one booster given at 365 days after the first vaccination.

e) Stability

Due to the fact that the inactivated vaccine is a relatively new product, not much data are available on the duration of its stability. Results have been obtained that demonstrate vaccine stability in excess of 3 years.

C3. African horse sickness subunit vaccine

AHSV 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 in different combination to immunise horses (24). However, this vaccine is not commercially available.

An experimental vaccine using crude cell extracts containing the three structural proteins was sufficient to obtain a complete protective immune response in horses challenged with AHS virulence virus (10^6 TCID₅₀). Viraemia was

not detectable in vaccinated horse (24). Further analysis of partially protective crude lysate revealed that only soluble VP2 was capable of inducing neutralising antibodies. A definition of neutralising sites of the VP2 virus protein of AHSV serotypes 3, 4 and 9 has been determined (2, 25, 36) and very recently, the full protection of horses immunised with a soluble recombinant VP2 protein (AHSV serotype 5) administered with saponin to a lethal AHSV challenge has been reported (32). Although further experiments need to be performed to estimate the duration of the immunity induced by these proteins, the data indicate the effectiveness of this candidate vaccine.

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NB: There are OIE Reference Laboratories for African horse sickness (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).