

Council Directive 2009/156/EC of 30 November 2009 on animal health conditions governing the movement and importation from third countries of equidae (codified version) (Text with EEA relevance)

[^{F1}ANNEX IV

AFRICAN HORSE SICKNESS DIAGNOSIS

Textual Amendments

- F1** Substituted by [Commission Implementing Decision \(EU\) 2016/1840 of 14 October 2016 amending Annex IV to Council Directive 2009/156/EC as regards methods for African horse sickness diagnosis \(notified under document C\(2016\) 6509\) \(Text with EEA relevance\).](#)

PART A

Serological tests

The serological method described hereinafter are enzyme-linked immunosorbent assays (ELISA) based on point 2 of Section B in Chapter 2.5.1 of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Edition 2016 as adopted by the World Assembly of Delegates of the OIE in May 2012.

The VP7 viral protein is an immuno-dominant major antigen of the African horse sickness virus (AHSV) and is conserved across the nine AHSV serotypes. Recombinant AHSV-VP7 proteins have been shown to be stable and innocuous and suitable to be used as antigens in ELISA procedures for determination of AHSV antibodies with a high degree of sensitivity and specificity (Laviada et al., 1992b⁽¹⁾; Maree and Paweska, 2005). The indirect ELISA and the blocking ELISA are the two AHS-VP7 ELISA tests suitable for serological diagnosis of African horse sickness (AHS).

1. **Indirect ELISA for the detection of antibodies to African horse sickness virus (AHSV)**

The conjugate used in this method is a horseradish peroxidase anti-horse gamma-globulin reacting with the serum of horses, mules and donkeys. The method described by Maree & Paweska (2005)⁽²⁾ uses protein G as conjugate that also reacts with zebra serum.

The antigen may be provided by the Centro de Investigación en Sanidad Animal (CISA), Spain, within 4 to 6 months of request.

1.1. *Test procedure*

1.1.1. Solid phase

1.1.1.1. Coat ELISA plates with recombinant AHSV-4 VP7 diluted in carbonate/bicarbonate buffer, pH 9,6. Incubate plates overnight at 4 °C.

1.1.1.2. Wash the plates five times with distilled water containing 0,01 % (v/v) Tween 20 (washing solution). Gently tap the plates onto absorbent material to remove any residual wash.

1.1.1.3. Block the plates with phosphate buffered saline (PBS) pH 7,2 + 5 % (w/v) skimmed milk (Nestlé Dry Skim MilkTM), 200 µl/well, for 1 hour at 37 °C.

1.1.1.4. Remove the blocking solution and gently tap the plates onto absorbent material.

1.1.2. Test samples

- 1.1.2.1. Serum samples to be tested, and positive and negative control sera, are diluted 1 in 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, make a twofold dilution series from 1 in 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.

- 1.1.2.2. Wash the plates five times with distilled water containing 0,01 % (v/v) Tween 20 (washing solution). Gently tap the plates onto absorbent material to remove any residual wash.

1.1.3. Conjugate

- 1.1.3.1. Dispense 100 µl/well of horseradish-peroxidase (HRP) -conjugated anti-horse gamma-globulin diluted in PBS + 5 % milk + 0,05 % Tween 20, pH 7,2. Incubate for 1 hour at 37 °C.

- 1.1.3.2. Wash the plates five times with distilled water containing 0,01 % (v/v) Tween 20 (washing solution). Gently tap the plates onto absorbent material to remove any residual wash.

1.1.4. Chromogen/Substrate

- 1.1.4.1. Add 200 µl/well of chromogen/substrate solution (10 ml of 80,6 mM DMAB (dimethyl aminobenzaldehyde) + 10 ml of 1,56 mM MBTH (3-methyl-2-benzo-thiazoline hydrazone hydrochlorid) + 5 µl H₂O₂).

Colour development is stopped by adding 50 µl of 3N H₂SO₄ after approximately 5 to 10 minutes (before the negative control begins to be coloured).

Other chromogens such as ABTS (2,2'-Azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]), TMB (tetramethyl benzidine), or OPD (ortho-phenyldiamine) can also be used.

- 1.1.4.2. Read the plates at 600 nm (or 620 nm).

1.2. Interpretation of the results

- 1.2.1. 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).

- 1.2.2. Test samples giving absorbance values lower than the cut-off are regarded as negative.

- 1.2.3. Test samples giving absorbance values greater than the cut-off + 0,15 are regarded as positive.

- 1.2.4. Test samples giving intermediate absorbance values are considered to be inconclusive and a second technique must be employed to confirm the result.

2. **Blocking ELISA for the detection of antibodies to African horse sickness virus (AHSV)**

The competitive blocking ELISA is designed to detect specific AHSV antibodies in sera from animals of any equine species, i.e. horses, donkeys, zebra and their crosses, preventing the problem of specificity experienced occasionally using the indirect ELISAs.

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The principle of the test is the blocking of the reaction between the recombinant VP7 protein absorbed to the ELISA plate and a conjugated AHS-VP7 specific monoclonal antibody (Mab). Antibody in the test sera will block the reaction between the antigen and the Mab resulting in a reduction in colour. Because the Mab is directed against the VP7, the assay will give a high level of sensitivity and specificity.

The competitive blocking ELISA is commercially available.

2.1. *Test procedure*

2.1.1. Solid Phase

2.1.1.1. Coat ELISA plates with 50-100 ng of recombinant AHSV-4 VP7 diluted in carbonate/bicarbonate buffer, pH 9,6. Incubate overnight at 4 °C.

2.1.1.2. Wash the plates three times with phosphate buffered saline (PBS) 0,1× containing 0,135 M NaCl and 0,05 % (v/v) Tween 20 (PBST). Gently tap the plates on to absorbent material to remove any residual wash.

2.1.2. Test samples and controls

2.1.2.1. Serum samples to be tested, and positive and negative control sera, are diluted 1 in 5 in diluent containing 0,35 M NaCl, 0,05 % (v/v) Tween 20 and 0,1 % Kathon, 100 µl per well. Incubate for 1 hour at 37 °C.

For titration, make a twofold dilution series of the test sera from 1 in 10 to 1 in 280 across 8 wells (100 µl/well), one serum per plate column, and do the same with positive and negative controls. Incubate for 1 hour at 37 °C.

2.1.2.2. Wash the plates five times with phosphate buffered saline (PBS) 0,1× containing 0,135 M NaCl and 0,05 % (v/v) Tween 20 (PBST). Gently tap the plates on to absorbent material to remove any residual wash.

2.1.3. Conjugate

2.1.3.1. Dispense 100 µl/well of horseradish peroxidase-conjugated Mab anti-VP7. In advance, this Mab has been diluted 1/5 000-1/15 000 in a 1/1 solution of StabiliZyme Select® Stabilizer (SurModics. Reference: SZ03) in distilled water. Incubate for 30 minutes at 37 °C.

2.1.3.2. Wash the plates five times with phosphate buffered saline (PBS) 0,1× containing 0,135 M NaCl and 0,05 % (v/v) Tween 20 (PBST). Gently tap the plates on to absorbent material to remove any residual wash.

2.1.4. Chromogen/Substrate

Add 100 µl/well chromogen/substrate solution, i.e. 1 ml of ABTS (2,2'-Azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) 5 mg/ml + 9 ml of substrate buffer (0,1 M Phosphate-Citrate buffer of pH 4 containing 0,03 % H₂O₂), and incubate for 10 minutes at room temperature. Colour development is stopped by adding 100 µl/well of 2 % (w/v) SDS (sodium dodecyl sulphate).

2.1.5. Reading

Read at 405 nm in an ELISA reader.

2.2. *Interpretation of the results*

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- 2.2.1. Determine the blocking percentage (BP) of each sample by applying the following formula, where 'Abs' stands for antibodies:

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

- 2.2.2. Samples showing a BP value higher than 50 % should be considered as positive for AHSV antibodies.
- 2.2.3. Samples showing a BP value lower than 45 % should be considered as negative for AHSV antibodies.
- 2.2.4. Samples showing a BP value between 45 % and 50 % should be considered as inconclusive and must be retested. If the result is again inconclusive, the animals should be retested on samples taken not earlier than two weeks after the sample which was considered to be inconclusive was taken.]

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- (1) [^{F1}Laviada M.D., Roy P. and Sanchez-Vizcaino J.M (1992b). Adaptation and evaluation of an indirect ELISA and immunoblotting test for African horse sickness antibody detection. In: Bluetongue, African Horse Sickness and Related Orbiviruses: Proceedings of the Second International Symposium. Walton T.E. & Osburn B.I., Eds. CRC Press, Boca Raton, Florida, USA, 646-650.]
- (2) [^{F1}Maree S. and Paweska J.T. (2005). Preparation of recombinant African horse sickness virus VP7 antigen via a simple method and validation of a VP7-based indirect ELISA for the detection of group-specific IgG antibodies in horse sera. J. Virol. Methods, 125 (1), 55-65.]

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