Section 3.6

Equidae

CHAPTER 3.6.5

EQUINE INFECTIOUS ANAEMIA (INFECTION WITH EQUINE INFECTIOUS ANAEMIA VIRUS)

SUMMARY

Equine infectious anaemia (*EIA*) *is a persistent viral infection of equids. The causative agent, EIA virus* (*EIAV*) *is a Lentivirus classified in the family* Retroviridae*, subfamily* Orthoretrovirinae, species Lentivirus equinfane*. Although EIA may be suspected by the presence of suggestive clinical signs and pathological lesions, confirmation of infection requires laboratory diagnosis and is usually by serology. Infected horses remain viraemic carriers for life and, with very rare exceptions, yield a positive serological test result. Although antibody levels fluctuate, EIAV infection generates a persistent antibody response. All equids older than 12 months that test seropositive are identified as virus carriers. In young equids less than 12 months of age, positive serological reactions can be due to maternal antibodies; therefore, the EIA status may have to rely solely on molecular techniques. As virus reservoirs, infected equids represent a transmission risk to other equids. The virus is primarily blood-borne. Biting flies are mechanical vectors for the virus in nature and infection is often spread via iatrogenic routes.*

Identification of the agent: *EIAV cannot be differentiated clinically from a number of other aetiological agents of haemolytic anaemic fever syndromes and systemic equine diseases. Diagnosis of EIAV infection is laboratory dependent and based on the demonstration of a specific antibody response, virus isolation, or detection of viral nucleic acid.*

Virus can be isolated by inoculating suspect blood onto leukocyte cultures prepared from susceptible horses. Recognition of infection in experimentally challenged horses may be made based on clinical signs, haematological changes, positive serological reactions and/or detection of the virus by molecular techniques. Successful virus isolation in equine monocyte-derived macrophages (*eMDMs*), *derived from peripheral blood mononuclear cells* (*PBMCs*), *need to be confirmed by the detection of specific EIA antigen, polymerase chain reaction-based techniques, or by the inoculation of culture fluids into susceptible horses. Virus isolation is rarely attempted due to the time, difficulty and expense involved.*

Serological tests: *Agar gel immunodiffusion* (*AGID*) *tests and enzyme-linked immunosorbent assays* (*ELISAs*), *are simple, reliable serological tests for the demonstration of EIAV infection. The AGID tests should be used to confirm positive ELISA results. An immunoblot may be used in case of discrepancy in the results. Antibody levels are highly variable and fluctuate due to the changing nature of the virus and the immune status of the infected subject. EIA antigens can be prepared from infected cell cultures or by using recombinant DNA technology. A variety of licensed and validated commercial test kits is available.*

The colloidal gold immunochromatographic strip (CGICG) test has demonstrated potential for the diagnosis of EIA.

Requirements for vaccines: *An attenuated live vaccine was developed in the early 1970s and used extensively in China (People's Rep. of) between 1975 and 1990. Numerous other methods of vaccine production have since been attempted with limited success. The strategy for EIA control has shifted from vaccination to quarantine to avoid the interference of vaccinal antibodies with diagnostic tests. There are no vaccines currently available.*

A. INTRODUCTION

1. Description of disease and aetiology

Equine infectious anaemia (EIA) occurs worldwide and causes significant losses to the equine industry. EIA is a WOAH notifiable disease. The infection, formerly known as swamp fever, is limited to equids including horses, donkeys, mules and hinny (Wang *et al.,* 2023), though studies on the presence of the infection in zebras are scarce. Many cases remain clinically inapparent. The disease is characterised by recurrent febrile episodes, thrombocytopenia, anaemia, rapid loss of weight, cachexia, jaundice, and oedema of the lower parts of the body. If death does not result from one of the acute clinical attacks, a chronic stage develops, and the infection tends to become inapparent. The incubation period is normally 1–3 weeks, but may be as long as 3 months or more. Some horses remain subclinical until they experience some form of stress (Harrold *et al*., 2020) or may never show noticeable clinical signs. In acute cases, lymph nodes, spleen and liver are hyperaemic and enlarged. Histologically, these organs are infiltrated with nests of immature lymphocytes and plasma cells. Kupffer cells in the liver often contain haemosiderin or erythrocytes. The enlarged spleen may be felt on rectal examination. Differential diagnoses include equine viral arteritis, *Anaplasma phagocytophilum, Piroplasmosis, leptospirosis, severe strongyliosis* and other causes of oedema, fever, anaemia, or thrombocytopaenia/ ecchymoses.

EIA is a persistent viral infection of equids. The causative agent, EIA virus (EIAV) is a *Lentivirus* classified in the family *Retroviridae,* subfamily *Orthoretrovirinae*, genus *Lentivirus*, species *Lentivirus equinfane*. Once a horse is infected with EIAV, its blood remains infectious for the remainder of its life and the horse can potentially transmit the infection to other horses. Transmission occurs by transfer of blood or contaminated secretions from an infected horse. In nature, spread of the virus is most likely via interrupted feeding of bloodsucking horseflies (*Tabanidae*) and stable flies (Dantas Malossi *et al.,* 2020) on an infected horse and then on susceptible horses. Transmission by haematophagous flies is mechanical as it does not involve EIAV replication in their tissues (Fideles Resende *et al.,* 2022). Transmission can also occur by the iatrogenic transfer of blood using contaminated blood products, needles, syringes, IV administration sets or other equipment. Infection can also be transmitted through the congenital route or body secretions, e.g. semen, sweat, urine (Romo-Sáenz *et al.,* 2021). No effective treatment or vaccine is currently available (Dantas Malossi *et al.,* 2020). The viral load is higher in the plasma of horses with clinical signs and the risk of transmission is higher from these animals than the apparently healthy carrier animals with a lower viral load. Studies in mules have demonstrated a lack of correlation between viral load and serological reactivity. Naturally infected asymptomatic mules with positive enzyme-linked immunosorbent assay (ELISA) but negative or equivocal agar gel immunodiffusion (AGID) test results may have high virus nucleic acid loads in the plasma and could potentially contribute to the maintenance and spread of infection.

EIAV is not considered a risk for human health. Laboratory manipulations should be carried out at an appropriate biosafety and containment level 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

AGID tests (Coggins *et al*., 1972) and ELISAs are accurate, reliable tests for the detection of EIA in horses, except for animals in the early stages of infection and foals of infected dams (United States Department of Agriculture [USDA], 2007). In other rare circumstances, misleading results may occur when the level of virus circulating in the blood during an acute episode of the disease is sufficient to bind available antibody, and if initial antibody levels never rise high enough to be detectable. Although the ELISA will detect antibodies somewhat earlier and at lower concentrations than the AGID test, positive ELISAs are confirmed using the AGID test. This is due to false-positive results that have been noted with indirect ELISAs. The AGID test is specific, thus has the advantage of distinguishing between EIA and non-EIA antigen–antibody reactions. Nonspecific reactions in AGID may occur with antigen derived from the spleen of infected animals or equine dermal cell cultures (https://www.atcc.org/products/ccl-57) that might contain other cellular or host-derived proteins with consequent nonspecific precipitation lines with antibodies present in the tested serum against non-EIAV antigens. Recombinant technology for the production of EIAV antigens can obviate AGID nonspecific reactions (Alvarez *et al*., 2007). Competitive

ELISA developed using specific monoclonal antibodies against a common epitope on viral P26 protein showed high specificity and sensitivity (Hu *et al*., 2023). A colloidal gold immunochromatographic (CGICG) fast test strip was developed with good specificity, sensitivity, stability, and repeatability, which provides a potential tool for point-of-care testing for the primary screening of EIAV antibodies (Zhang *et al*., 2024). Discrepancies between testing methods or tests with questionable results can be further evaluated by immunoblot testing (Issel *et al.,* 2013).

Table 1. Test methods available for the diagnosis of equine infectious anaemia and their purpose

Method	Purpose					
	Population freedom from infection[a]	**Individual animal freedom from infection prior to movement[b]**	**Contribute to eradication policies[c]**	**Confirmation of clinical cases[d]**	**Prevalence of infection – surveillance[e]**	**Immune status in individual animals or populations (post-vaccination)**
Identification of the agent[f]						
PCR	□□□	☑□□/□□□	□□□	☑□□/□□□	☑□□/□□□	□□□
Virus isolation	□□□	□□□	□□□	☑□□	□□□	□□□
Detection of immune response						
AGID	☑☑□	☑☑□	☑☑□	☑☑□	☑☑□	□□□
ELISA	☑☑☑	☑☑☑	☑☑☑	☑☑□	☑☑☑	□□□

Key: ☑☑☑ = recommended for this purpose; ☑☑□ = recommended but has limitations;
☑□□ = suitable in very limited circumstances; □□□ = not appropriate for this purpose.
PCR = polymerase chain reaction; AGID = agar gel immunodiffusion; ELISA = enzyme-linked immunosorbent assay.

[a] See Appendix 1 of this chapter for justification table for the scores given to the tests for this purpose.
[b] See Appendix 2 of this chapter for justification table for the scores given to the tests for this purpose.
[c] See Appendix 3 of this chapter for justification table for the scores given to the tests for this purpose.
[d] See Appendix 4 of this chapter for justification table for the scores given to the tests for this purpose.
[e] See Appendix 5 of this chapter for justification table for the scores given to the tests for this purpose.
[f] A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agent

1.1. Virus isolation and identification

Virus isolation is usually not necessary to make a diagnosis.

Isolation of the virus from suspect horses may be made by inoculating their blood (plasma, serum, leukocytes) or spleen homogenates onto equine monocyte-derived macrophages (eMDMs) derived from peripheral blood mononuclear cells (PBMCs) cultures prepared from horses free of infection (Fidalgo-Carvalho *et al.,* 2009). Virus production in cultures can be

confirmed by detection of specific EIA antigen by ELISA, or by molecular tests (Cook *et al.*, 2002; Dong *et al.*, 2012). Virus isolation is rarely attempted because of the difficulty of growing horse leukocyte cultures and poor adaptability of some strains to cell culture (Ma *et al.*, 2014).

1.2. Polymerase chain reaction

A nested polymerase chain reaction (PCR) assay to detect EIA proviral DNA from the peripheral blood of horses has been described (Nagarajan & Simard,2007). The nested PCR method is based on primer sequences from the gag region of the proviral genome. It has proven to be a sensitive technique to detect field strains of EIAV in white blood cells of EIA infected horses; the lower limit of detection is typically around 10 genomic copies of the target DNA (Nagarajan & Simard, 2007). A real-time reverse-transcriptase PCR assay has also been described (Cook *et al.,* 2002). Other real-time PCR methods are based on primer sequences from the exon 1 tat genomic portion (118 bp) that has a limit of detection of the EIAV tat RNA transcript of 1 genomic copy (Scicluna *et al.*, 2013). Another real-time PCR amplifies a fragment between the tat and the gag genes (Li *et al.*, 2023). To increase the diagnostic sensitivity, PCR methods for detecting viral RNA and proviral DNA should be used. To confirm the results of these very sensitive assays, it is recommended that duplicate samples of each diagnostic specimen be processed. Because of the risk of cross contamination, it is also important that proper procedures are followed (see Chapter 1.1.5 *Quality management in veterinary testing laboratories* and Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*). It should be noted that primer mismatches with circulating virus, possibly caused by the high rate of mutation in the virus, may cause a failure of PCR to detect virus (Li *et al.*, 2023; Scicluna *et al.*, 2013).

The following are some of the circumstances where the PCR assay maybe used for the detection of EIAV infection in horses:

i) Conflicting results on serologic tests;

ii) Suspected infection but negative or questionable serologic results;

iii) Complementary test to serology for the confirmation of positive results;

iv) Confirmation of early infection, when the serum antibodies to EIAV are not detectable;

v) In addition to serology, to ensure that horses that are used for antiserum or vaccine production or as blood donors are free of EIAV;

vi) Confirmation of the status of a foal from an infected mare.

1.3. Gene sequencing

Genetic characterisation of EIAV strains is useful in epidemiological investigations to possibly infer virus origin and monitor spread. Targeted sequence enrichment and next generation sequencing allow direct sequencing of the EIAV whole genome without cloning or amplification steps (Deshiere *et al.,* 2019).

2. Serological tests

Due to the persistence of EIAV in infected equids, detection of serum antibody to EIAV confirms the diagnosis of EIAV infection. An efficient diagnostic algorithm in terms of sensitivity and specificity can be adopted, such as the one based on a three-tiered diagnostic system (Issel *et al.*, 2013; Scicluna *et al.*, 2013). This procedure involves initial screening by ELISA (Tier 1) with test-positive samples confirmed by the AGID (Tier 2) and, in the case of ELISA positive/AGID negative results, final verification by Immunoblotting (IB) (Tier 3).

2.1. Agar gel immunodiffusion test

The AGID test detects precipitating antibody produced in response to EIAV infection. Specific reactions are indicated by precipitation lines between the EIA antigen and the test serum and confirmed by their identity with the reaction between the antigen and the positive standard serum.

Reagents for AGID are available commercially from several companies. Alternatively, AGID antigen and reference serum may be prepared as described below.

2.1.1. Preparation of antigen

Specific EIA antigen may be prepared from the spleen of acutely infected horses (Coggins *et al.*, 1973), from infected equine tissue culture, from a persistently infected canine thymus cell line, or from proteins expressed in bacteria or baculovirus using the recombinant DNA technique. Preparation from infected cultures or from recombinant DNA techniques gives a more uniform result than the use of spleen cells and allows for better standardisation of reagents.

Equine fetal kidney or dermal cells or canine thymus cells are infected with a strain of EIAV adapted to grow in tissue culture (American Type Culture Collection, or Chinese strain adapted to equine fetal dermal cells). Virus is collected from cultures by precipitation with 8% polyethylene glycol or by pelleting by ultracentrifugation. The diagnostic antigen, p26, is released from the virus by treatment with detergent or ether. EIAV core proteins, expressed in bacteria, i.e. *E. coli* or baculovirus (Alvarez *et al.*, 2007; Scicluna *et al.,* 2019), are commercially available and find practical use as high quality antigens for serological diagnosis (Bannai *et al.* 2023).

The p26 is an internal structural protein of the virus that is coded for by the *gag* gene. The p26 is more antigenically stable among EIAV strains than the virion glycoproteins gp45 and gp90. There is evidence of strain variation in the p26 amino acid sequence; however, there is no evidence to indicate that this variation influences any of the serological diagnostic tests.

2.1.2. Preparation of standard antiserum

A known positive serum may be collected from a horse previously experimentally or naturally infected with EIAV. This serum should yield a single dense precipitation line that is specific for EIA, as demonstrated by a reaction of identity in comparison with a known standard positive reference serum. It is essential to balance the antigen and antibody concentrations to ensure the optimal sensitivity of the test. Reagent concentrations should be adjusted by cross-testing serial dilutions of antigen and serum to obtain a narrow precipitation line approximately equidistant between the two wells containing the antigen and the standard positive reference serum (Chapter 2.2.6 *Selection and use of reference samples and panels*). A list of WOAH-approved International Standard Reagents is also available[1]).

2.1.3. Test procedure (Association Française de Normalisation [AFNOR], 2000; Coggins *et al.*, 1973)

i) Immunodiffusion reactions are carried out in a layer of agar in plastic Petri dishes as glass dishes can result in slippage. For Petri dishes that are 100 mm in diameter, 15–17 ml of 1% Noble agar in 0.145 M borate buffer (9 g H_3BO_3, plus 2 g NaOH per litre), pH 8.6 (± 0.2) is used. A metal punch is used to create several “rosettes,” each of six wells surrounding a centre well of the same diameter. The wells are 5.3 mm in diameter and 2.4 mm apart. Each well must contain the same volume of reagent and should be completely but not over-filled.

ii) The antigen is placed in the central well and the standard antiserum is placed in alternate exterior wells. Serum samples for testing are placed in the remaining three wells.

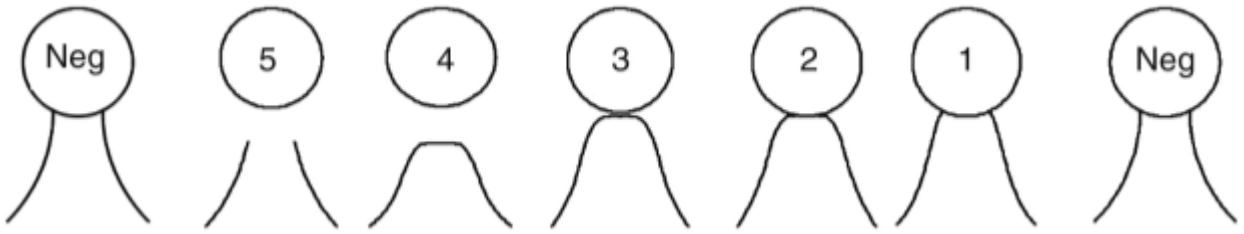
iii) The dishes are maintained at room temperature in a humid environment (18°C–26°C recommended).

iv) After 24 and 48 hours the precipitation reactions are examined over a narrow beam of intense, oblique light and against a black background, with also the help of a magnification. The reference lines should be clearly visible at 24 hours, and at that time, any test sera that are strongly positive may also have formed lines of identity with those between the standard reagents. A weakly positive reaction may take 48 hours to form and is indicated by a slight bending of the standard serum precipitation line between the antigen well and the test serum well. For EIA AGID, the bending caused by a weak positive reaction looks like a very small hook or rounding into the sample well. Sera with high precipitating antibody titres will form a complete line of identity or may form broader precipitin bands that break into the sample well or tend to be diffuse (see Figure 1). Such reactions can be confirmed as specific for EIA by dilution at 1/2 and subsequent serial dilutions prior to retesting; these then give a more distinct line of identity. Sera devoid of EIA antibody will not form precipitation lines and will have no effect on the reaction lines of the standard reagents. Nonspecific precipitation lines may occur. These nonspecific lines can cross the control lines, typically showing no line of identity with the control lines.

v) Interpretation of the results: Horses that are in the early stages of an infection may not give a positive serological reaction in an AGID test. Such animals should be bled again after 3–4 weeks, or, in case of clinical signs at least a week after their appearance. To make a diagnosis in a young foal, it may be necessary to determine the antibody

status of the dam. If the mare passes EIA antibody to the foal through colostrum, then a period of 6 months or longer after birth must be allowed for the maternally-derived antibody to wane. Sequential testing of the foal at monthly intervals may be useful to observe the decline in maternal antibody. To conclude that the foal is not infected, a negative result must be obtained (following an initial positive result) at least 3 months after separating the foal from contact with the EIA positive mare or any other positive horse. It should be noted that maternal antibodies can often be detected for up to 12 months of age, therefore alternative diagnostic methods should be considered, for example PCR could be used to determine the presence/absence of EIA virus in the blood of the foal.

Fig. 1. Reactions in AGID test for EIAV.



From: Issel et al. (2013).

2.2. Enzyme-linked immunosorbent assay

Several diagnostic test kits for EIA, including AGID and ELISA, are licensed in various countries for the diagnosis of equine infectious anaemia and are available internationally (Hu *et al.*, 2023; Nardini *et al.*, 2017). The ELISAs generally target antibody produced against the p26 core protein antigen but may also have a second target antibody produced against the gp45 antigen. These antigens may also be synthetic fusion proteins or recombinant antigens. Typical ELISA protocols are used. Both indirect and competitive ELISAs are used for disease surveillance. If commercial ELISA materials are not available, a non-competitive ELISA using p26 antigen purified from cell culture material may be employed.

A positive test result by ELISA should be confirmed using the AGID test because false-positive results have been noted with the ELISA. The results can also be confirmed by the immunoblotting technique. A standard antiserum for immunodiffusion, which contains detectable antibody, is available from the WOAH Reference Laboratories[2]. This standard should not be used as the reference for minimum detection limits for the ELISA reaction. Uniform methods for EIA control have been published (USDA, 2007).

2.3. Immunoblotting test

Immunoblotting (IB) is characterised by both high sensitivity and specificity and is the alternative confirmatory assay for ELISA/AGID discordant results. In the IB, the immunological reactivity to EIAV antigens, as in the case of using the whole virus, p26, gp45 and gp90 adsorbed on the IB membrane, can be used to define the serological status of the animal for EIA: a p26 positive band together with at least one of the other antigen defines a subject as serologically positive for EIAV (Issel *et al.*, 2013; Scicluna *et al.* 2019). This test is not commercially available and has not been the subjected to an international proficiency test; this test is only available at WOAH Reference Laboratory in Italy.

2.4. Colloidal gold immunochromatographic test

Colloidal gold immunochromatographic test (CGICG) is a method that has been widely used for disease screening and surveillance, especially in human diseases such as COVID-19. In the CGICG methods, the P26 and gp45 are major antigens used to detect EIAV antibodies. The CGICG test for EIAV antibody detection showed high sensitivity and specificity, and can be concluded within 15 minutes in on-site detection (Alvarez *et al.,* 2010; Zhang *et al.,* 2024), thereby potentially providing an alternative method for disease surveillance.

C. REQUIREMENTS FOR VACCINES

Inactivated and subunit EIAV vaccines were tested in different laboratories and proved to protect infections of homologous prototype strains only. An attenuated live vaccine, developed in the early 1970s, was extensively used in China (People's Rep. of) between 1975 and 1990 and was effective in controlling the prevalence of EIA. With low prevalence since 1990, the strategy for EIA control has shifted from vaccination to quarantine to avoid the interference of vaccine antibodies with diagnostic tests that are not yet capable to differentiate vaccinated and infected subjects.

Although no safety concerns arose with the use of attenuated EIAV vaccine in China, it should be noted that, like other lentiviruses, EIAV is highly mutable and can integrate into host genomes (Lin *et al*., 2020; Liu *et al*.,2017). The use of a live EIAV vaccine should be considered only after a thorough risk assessment.

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NB: There are WOAH Reference Laboratories for equine infectious anaemia:
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3)
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests and reagents for equine infectious anaemia

Appendix 1: Equine infectious anaemia
Intended purpose of test: population freedom from infection

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

PCR □□□	Blood Plasma Organs (spleen, liver) Exon 1 tat Blood samples LTR-tat	For Exon 1 tat: Analytical sensitivity (LOD) = 1 copy target/µl Analytical specificity = 44 exon 1 tata amplicons sequenced For LTR-tat: Analytical sensitivity (LOD) = 10 copy target/µl Analytical specificity = 40 LTR-tat amplicons sequenced	For Exon 1 tat: 5 Mules plasma (all positive) 37 positive equine bloods 16 positive equine plasma For LTR-tat: 53 blood samples (40 positive sequenced and 13 negative) See reference	See reference	For Exon 1 tat and LTR tat 1. High analytical sensitivity 2. High analytical specificity	For Exon 1 tat and LTR tat 1. Relatively low turnover 2. High costs 3. Requires specific laboratory equipment 4. Requires skilled laboratory personnel 5. Potential low diagnostic specificity due to the high genetic variability of the virus	Scicluna *et al.* (2013)
AGID ☑☑□	Serum recombinant p26	Reference test: IB Specificity 100% Sensitivity 59.4%	548 samples positive in a screening ELISA subsequently analysed by AGID and IB at the WOAH RL, Italy	To be published	1. High specificity 2. High sensitivity in case of populations not previously tested 3. Low cost 4. Minimal laboratory equipment required	1. Low sensitivity in case of early infection or low antibody levels 2. Low turnover 3. Results are not readily available 4. In case of low levels of reactivity, interpretation of results depends on technical expertise	

ELISA ☑☑☑	Serum, recombinant p26, chimeric recombinant p26/gp45	Reference test were: AGID, IB **Recombinant p26:** Dsp: 73.2% (CI: 67.6–78.1%) Dse: 100% (CI: 91.6–100%) 50% inhibition as determined by ROC analysis. **Recombinant p26/gp45:** Dsp: 99.3% (CI: 96.8-99.8%) Dse: 100% (CI: 100-100%) 50% inhibition as determined by ROC analysis. **bELISA:** Reference tests were AGID Dsp = 100% Dse = 97.0% Accuracy 98.3%	**Recombinant p26:** 324 Field sera analysed by immunoblot (52 positive, 272 negative). 96 Sera of horses experimentally vaccinated 1,102 field sera analysed by AGID test (857 positive and 245 negative) Intralaboratory test with nine laboratories **Recombinant p26/gp45:** 96 Sera of horses experimentally vaccinated 615 field sera analysed by AGID test **bELISA:** 1129 negative samples were taken from different farms in China which were free from EIA 18 positive sera, 45 negative sera, and 353 test serum samples from Argentina 3 standard positive sera 15,900 serum samples of either horses or donkeys collected from different provinces of China	See reference	1. High sensitivity 2. Serological ELISAs are commercially available in many countries, together with in house ELISAs (see references) 3. A comparative analysis of some commercial and in house ELISAs reported that their diagnostic performances are comparable (see reference) 4. Rapid to carry out with an immediate result output 5. Low cost 6. Target antigen used is usually highly conserved 7. High turnover 8. No cross reactivity with other known viruses	1. Specific laboratory equipment required 2. Considering the diagnostic specificity, positive samples should be confirmed using AGID and, in case of discordant results, in IB 3. Generally, low sensitivity during early stages of infection 4. False-positive results have been noted with some ELISAs	Nardini *et al.* (2016) Nardini *et al.* (2017) Scicluna *et al.* (2018) Naves *et al.* (2019) Dominguez *et al.* (2021) Hu *et al.* (2023) Russi *et al.* (2023)

Appendix 2: Equine infectious anaemia

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

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

PCR ☑□□/□□□	Blood Plasma Organs (spleen, liver) Exon 1 tat Blood samples LTR-tat	For Exon 1 tat: Analytical sensitivity (LOD) = 1 copy target/µl Analytical specificity = 44 exon 1 tata amplicons sequenced For LTR-tat: Analytical sensitivity (LOD) = 10 copy target/µl Analytical specificity = 40 LTR-tat amplicons sequenced	For Exon 1 tat: 5 Mules plasma (all positive) 37 positive equine bloods 16 positive equine plasma For LTR-tat: 53 blood samples (40 positive sequenced and 13 negative) See reference	See reference	For Exon 1 tat and LTR tat 1. High analytical sensitivity 2. High analytical specificity	For Exon 1 tat and LTR tat 1. Requires specific laboratory equipment 2. Skilled personnel required 3. Potential low diagnostic specificity due to the high genetic variability of the virus	Scicluna *et al.* (2013)
AGID ☑☑□	Serum recombinant p26	Reference test: IB Specificity 100% Sensitivity 59.4%	548 samples positive in a screening ELISA subsequently analysed by AGID and IB at the WOAH RL, Italy	To be published	1. High specificity 2. Low cost 3. Minimal laboratory equipment required	1. Low sensitivity in case of early infection or low antibodies levels 2. Results are not readily available 3. In case of low levels of reactivity, interpretation of results depends on technical expertise	

ELISA ☑☑☑	Serum recombinant p26 chimeric recombinant p26/gp45	Reference test were: AGID, IB **Recombinant p26:** Dsp: 73.2% (CI: 67.6–78.1%) Dse: 100% (CI: 91.6–100%) 50% inhibition as determined by ROC analysis. **Recombinant p26/gp45:** Dsp: 99.3% (CI: 96.8-99.8%) Dse: 100% (CI: 100-100%) 50% inhibition as determined by ROC analysis. **bELISA:** Reference tests were AGID Dsp = 100% Dse = 97.0% Accuracy 98.3%	**Recombinant p26:** 1. 324 Field sera analysed by immunoblot (52 positive, 272 negative). 2. 96 Sera of horses experimentally vaccinated 3. 1,102 field sera analysed by AGID test (857 positive and 245 negative) 4. Intralaboratory test with nine laboratories **Recombinant p26/gp45:** 1. 96 Sera of horses experimentally vaccinated 3. 615 field sera analysed by AGID test **bELISA:** 1129 negative samples were taken from different farms in China which were free from EIA 18 positive sera, 45 negative sera, and 353 test serum samples from Argentina 3 standard positive sera 15,900 serum samples of either horses or donkeys collected from different provinces of China (People's Rep. of)	See reference	1. High sensitivity 2. Serological ELISAs are commercially available in many countries, together with in house ELISAs (see references) 3. A comparative analysis of some commercial and in house ELISAs reported that their diagnostic performances are comparable (see reference) 4. Rapid to carry out with an immediate result output 5. Low cost 6. Target antigen used is usually highly conserved 7. High turnover 8. No cross reactivity with other known viruses	1. Specific laboratory equipment required 2. Considering the diagnostic specificity, positive samples should be confirmed using AGID and, in case of discordant results, in IB 3. Generally, low sensitivity during early stages of infection 4. False-positive results have been noted with some ELISAs	Nardini *et al.* (2016) Nardini *et al.* (2017) Scicluna *et al.* (2018) Naves *et al.* (2019) Dominguez *et al.* (2021) Hu *et al.* (2023) Russi *et al.* (2023)

Appendix 3: Equine infectious anaemia

Intended purpose of test: contribute to eradication policies

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
PCR □□□	Blood Plasma Organs (spleen, liver) Exon 1 tat Blood samples LTR-tat	For Exon 1 tat: Analytical sensitivity (LOD) = 1 copy target/µl Analytical specificity = 44 exon 1 tata amplicons sequenced For LTR-tat: Analytical sensitivity (LOD) = 10 copy target/µl Analytical specificity = 40 LTR-tat amplicons sequenced	For Exon 1 tat: 5 Mules plasma (all positive) 37 positive equine bloods 16 positive equine plasma For LTR-tat: 53 blood samples (40 positive sequenced and 13 negative) See reference	See reference	For Exon 1 tat and LTR tat 1. High analytical sensitivity 2. High analytical specificity 3. Historical experimental infection studies in susceptible equids indicated that positive results correlated with infectivity	For Exon 1 tat and LTR tat 1. Relatively low turnover 2. High costs 3. Requires specific laboratory equipment 4. Requires skilled laboratory personnel 5. Potential low diagnostic specificity due to the high genetic variability of the virus	Scicluna *et al.* (2013)
AGID ☑☑□	Serum recombinant p26	Reference test: IB Specificity 100% Sensitivity 59.4%	548 samples positive in a screening ELISA subsequently analysed by AGID and IB at the WOAH RL, Italy	To be published	1. High specificity 2. High sensitivity in the initial stages of the intended purpose 3. Low cost 4. Minimal laboratory equipment required	1. Low sensitivity in case of early infection or low antibody levels 2. Low turnover 3. Results not readily available 4. In case of low levels of reactivity, interpretation of results depends on technical expertise	

ELISA ☑☑☑	Serum recombinant p26 chimeric recombinant p26/gp45	Reference test were: AGID, IB **Recombinant p26:** Dsp: 73.2% (CI: 67.6–78.1%) Dse: 100% (CI: 91.6–100%) 50% inhibition as determined by ROC analysis. **Recombinant p26/gp45:** Dsp: 99.3% (CI: 96.8-99.8%) Dse: 100% (CI: 100-100%) 50% inhibition as determined by ROC analysis. **bELISA:** Reference tests were AGID Dsp = 100% Dse = 97.0% Accuracy 98.3%	**Recombinant p26:** 1. 324 Field sera analysed by immunoblot (52 positive, 272 negative). 2. 96 Sera of horses experimentally vaccinated 3. 1,102 field sera analysed by AGID test (857 positive and 245 negative) 4. Intralaboratory test with 9 laboratories **Recombinant p26/gp45:** 1. 96 Sera of horses experimentally vaccinated 2. 615 field sera analysed by AGID test **bELISA:** 1129 negative samples were taken from different farms in China which were free from EIA 18 positive sera, 45 negative sera, and 353 test serum samples from Argentina 3 standard positive sera 15,900 serum samples of either horses or donkeys collected from different provinces of China (People's Rep. of)	See reference	1. High sensitivity 2. Serological ELISAs are commercially available in many countries, together with in house ELISAs (see references) 3. A comparative analysis of some commercial and in house ELISAs reported that their diagnostic performances are comparable (see reference) 4. Rapid to carry out with an immediate result output 5. Low cost 6. Target antigen used is usually highly conserved 7. High turnover 8. No cross reactivity with other known viruses	1. Specific laboratory equipment required 2. Considering the diagnostic specificity, positive samples should be confirmed using AGID and, in case of discordant results, in IB 3. Generally, low sensitivity during early stages of infection 4. False-positive results have been noted with some ELISAs	Nardini *et al.* (2016) Nardini *et al.* (2017) Scicluna *et al.* (2018) Naves *et al.* (2019) Dominguez *et al.* (2021) Hu *et al.* (2023) Russi *et al.* (2023)

Appendix 4: Equine infectious anaemia

Intended purpose of test: confirmation of clinical cases

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

PCR ☑□□/□□□	Blood Plasma Organs (spleen, liver) Exon 1 tat Blood samples LTR-tat	For Exon 1 tat: Analytical sensitivity (LOD) = 1 copy target/µl Analytical specificity = 44 exon 1 tata amplicons sequenced For LTR-tat: Analytical sensitivity (LOD) = 10 copy target/µl Analytical specificity = 40 LTR-tat amplicons sequenced	For Exon 1 tat: 5 Mules plasma (all positive) 37 positive equine bloods 16 positive equine plasma For LTR-tat: 53 blood samples (40 positive sequenced and 13 negative) See reference	See reference	For Exon 1 tat and LTR tat 1. High analytical sensitivity 2. High analytical specificity	For Exon 1 tat and LTR tat 1. Relatively low turnover 2. High costs 3. Requires specific laboratory equipment 4. Requires skilled laboratory personnel 5. Potential low diagnostic specificity due to the high genetic variability of the virus	Scicluna *et al.* (2013
Virus isolation ☑□□	Plasma, serum, leukocytes or spleen homogenates. EIA virus	Data not available	Data not available	Data not available	Useful for the study of field strains	1. Low sensitivity 2. Skilled personnel required 3. Time consuming, not suitable for high turnover	Fidalgo-Carvalho *et al.* (2009) Ma *et al.* (2014)
AGID ☑☑□	Serum recombinant p26	Reference test: IB Specificity 100% Sensitivity 59.4%	548 samples positive in a screening ELISA subsequently analysed by AGID and IB at the WOAH RL, Italy	To be published	1. High specificity 2. High sensitivity in the initial stages of the intended purpose 3. Low cost 4. Minimal laboratory equipment required	1. Low sensitivity in case of early infection or low antibody levels 2. Low turnover 3. Results not readily available 4. In case of low levels of reactivity, interpretation of results depends on technical expertise 5. a single antibody test at one point in time may not detect recent exposure (i.e. a positive result can confirm the presence of infection, but a negative result may require re-sampling	Nardini *et al.* (2017) Cullinane *et al.* (2007)

ELISA ☑☑□	Serum recombinant p26 chimeric recombinant p26/gp45	Reference test were: AGID, IB **Recombinant p26:** Dsp: 73.2% (CI: 67.6–78.1%) Dse: 100% (CI: 91.6–100%) 50% inhibition as determined by ROC analysis. **Recombinant p26/gp45:** Dsp: 99.3% (CI: 96.8-99.8%) Dse: 100% (CI: 100-100%) 50% inhibition as determined by ROC analysis. **bELISA:** Reference tests were AGID Dsp = 100% Dse = 97.0% Accuracy 98.3%	**Recombinant p26:** 1. 324 Field sera analysed by immunoblot (52 positive, 272 negative). 2. 96 Sera of horses experimentally vaccinated 3. 1,102 field sera analysed by AGID test (857 positive and 245 negative) 4. Intralaboratory test with 9 laboratories **Recombinant p26/gp45:** 1. 96 Sera of horses experimentally vaccinated 3. 615 field sera analysed by AGID test **bELISA:** 1129 negative samples were taken from different farms in China which were free from EIA 18 positive sera, 45 negative sera, and 353 test serum samples from Argentina 3 standard positive sera 15,900 serum samples of either horses or donkeys collected from different provinces of China (People's Rep. of)	See reference	1. High sensitivity 2. Serological ELISAs are commercially available in many countries, together with in house ELISAs (see references) 3. A comparative analysis of some commercial and in house ELISAs reported that their diagnostic performances are comparable (see reference) 4. Rapid to carry out with an immediate result output 5. Low cost 6. Target antigen used is usually highly conserved 7. High turnover 8. No cross reactivity with other known viruses	1. Specific laboratory equipment required 2. Considering the diagnostic specificity, positive samples should be confirmed using AGID and, in case of discordant results, in IB 3. Generally, low sensitivity during early stages of infection 4. False-positive results have been noted with some ELISAs 5. a single antibody test at one point in time may not detect recent exposure (i.e. a positive result can confirm the presence of infection, but a negative result may require re-sampling	Nardini *et al.* (2016) Nardini *et al.* (2017) Cullinane *et al.* (2007) Scicluna *et al.* (2018) Naves *et al.* (2019) Dominguez *et al.* (2021) Hu *et al.* (2023) Russi *et al.* (2023)

Appendix 5: Equine infectious anaemia

Intended purpose of test: prevalence of infection – surveillance

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

PCR ☑□□/□□□	Blood Plasma Organs (spleen, liver) Exon 1 tat Blood samples LTR-tat	For Exon 1 tat: Analytical sensitivity (LOD) = 1 copy target/µl Analytical specificity = 44 exon 1 tata amplicons sequenced For LTR-tat: Analytical sensitivity (LOD) = 10 copy target/µl Analytical specificity = 40 LTR-tat amplicons sequenced	For Exon 1 tat: 5 Mules plasma (all positive) 37 positive equine bloods 16 positive equine plasma For LTR-tat: 53 blood samples (40 positive sequenced and 13 negative) See reference	See reference	For Exon 1 tat and LTR tat 1. High analytical sensitivity 2. High analytical specificity	For Exon 1 tat and LTR tat 1. Relatively low turnover 2. High costs 3. Requires specific laboratory equipment 4. Requires skilled laboratory personnel 5. Potential low diagnostic specificity due to the high genetic variability of the virus	Scicluna *et al.* (2013
AGID ☑☑□	Serum recombinant p26	Reference test: IB Specificity 100% Sensitivity 59.4%	548 samples positive in a screening ELISA subsequently analysed by AGID and IB at the WOAH RL, Italy	To be published	1. High specificity 2. High sensitivity in the initial stages of the intended purpose 3. Low cost 4. Minimal laboratory equipment required	1. Low sensitivity in case of early infection or low antibody levels 2. Low turnover 3. Results not readily available 4. In case of low levels of reactivity, interpretation of results depends on technical expertise	

ELISA ☑☑☑	Serum recombinant p26 chimeric recombinant p26/gp45	Reference test were: AGID, IB **Recombinant p26:** Dsp: 73.2% (CI: 67.6–78.1%) Dse: 100% (CI: 91.6–100%) 50% inhibition as determined by ROC analysis. **Recombinant p26/gp45:** Dsp: 99.3% (CI: 96.8-99.8%) Dse: 100% (CI: 100-100%) 50% inhibition as determined by ROC analysis. **bELISA:** Reference tests were AGID Dsp = 100% Dse = 97.0% Accuracy 98.3%	**Recombinant p26:** 1. 324 Field sera analysed by immunoblot (52 positive, 272 negative). 2. 96 Sera of horses experimentally vaccinated 3. 1,102 field sera analysed by AGID test (857 positive and 245 negative) 4. Intralaboratory test with nine laboratories **Recombinant p26/gp45:** 1. 96 Sera of horses experimentally vaccinated 3. 615 field sera analysed by AGID test **bELISA:** 1129 negative samples were taken from different farms in China which were free from EIA 18 positive sera, 45 negative sera, and 353 test serum samples from Argentina 3 standard positive sera 15,900 serum samples of either horses or donkeys collected from different provinces of China (People's Rep. of)	See reference	1. High sensitivity 2. Serological ELISAs are commercially available in many countries, together with in house ELISAs (see references) 3. A comparative analysis of some commercial and in house ELISAs reported that their diagnostic performances are comparable (see reference) 4. Rapid to carry out with an immediate result output 5. Low cost 6. Target antigen used is usually highly conserved 7. High turnover 8. No cross reactivity with other known viruses	1. Specific laboratory equipment required 2. Considering the diagnostic specificity, positive samples should be confirmed using AGID and, in case of discordant results, in IB 3. Generally, low sensitivity during early stages of infection 4. False-positive results have been noted with some ELISAs	Nardini *et al.* (2016) Nardini *et al.* (2017) Scicluna *et al.* (2018) Naves *et al.* (2019) Dominguez *et al.* (2021) Hu *et al.* (2023) Russi *et al.* (2023)

[1] https://www.woah.org/en/what-we-offer/veterinary-products/reference-reagents/
[2] https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

NB: First adopted in 1989. Most recent updates adopted in 2025.