EQUINE INFECTIOUS ANAEMIA

SUMMARY

Equine infectious anaemia (EIA) is a persistent viral infection of equids. The causative agent, EIA virus (EIAV) is a lentivirus in the family Retroviridae, *subfamily* Orthoretrovirinae. *Other members of the genus* Lentivirus *include: bovine immunodeficiency virus; caprine arthritis encephalitis virus; feline immunodeficiency virus; human immunodeficiency virus 1; human immunodeficiency virus 2; simian immunodeficiency virus and maedi/visna virus. Although EIA may be suspected on the basis of clinical signs and pathological lesions, confirmation of infection requires further serological and molecular-based testing. Infected horses remain viraemic carriers for life and, with very rare exceptions, yield a positive serological test result. Although antibody levels fluctuate, EIA 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 are 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: Virus can be isolated by inoculating suspect blood into a susceptible horse or onto leukocyte cultures prepared from susceptible horses. Recognition of infection in experimentally challenged horses may be made on the basis of clinical signs, haematological changes, positive serological reactions and/or detection of the virus by molecular techniques. Successful virus isolation in horse leukocyte cultures is confirmed by the detection of specific EIA antigen, by immunofluorescence assay, 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 test should be used to confirm positive ELISA results. Antibody levels are highly variable, and fluctuate due to the changing nature of the virus. EIA antigens can be prepared from infected tissue cultures or by using recombinant DNA technology. A variety of licensed and validated commercial test kits is available.

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 have since been attempted with variable results. 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

Equine infectious anaemia (EIA) occurs world-wide. The infection, formerly known as swamp fever, is limited to equids. Many cases remain clinically unapparent. The disease is characterised by recurrent febrile episodes, thrombocytopenia, anaemia, rapid loss of weight 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. 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 (Chapter 3.6.10), *Anaplasma phagocytophilum,* and other causes of oedema, fever, anaemia, or thrombocytopaenia/ecchymoses.

EIA virus (EIAV) is in the genus *Lentivirus* in the family *Retroviridae*, subfamily *Orthoretrovirinae*. Other members of the genus include: bovine immunodeficiency virus; caprine arthritis encephalitis virus; feline immunodeficiency virus; human immunodeficiency virus 1; human immunodeficiency virus 2; simian immunodeficiency virus; and maedi/visna virus.

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 (Cheevers & McGuire, 1985). Transmission occurs by transfer of blood or contaminated secretion from an infected horse. In nature, spread of the virus is most likely via interrupted feeding of bloodsucking horseflies (*Tabanidae*) on a clinically ill horse and then on susceptible horses. Transmission can also occur by the iatrogenic transfer of blood through the use of contaminated blood products, needles, syringes, IV administration sets or other equipment. *In utero* infection of the fetus may occur (Kemen & Coggins, 1972). The virus titre is higher in horses with clinical signs and the risk of transmission is higher from these animals than the carrier animals with a lower virus titre. However, studies in mules indicate infected animals with positive enzyme-linked immunosorbent assay (ELISA) and indeterminate agar gel immunodiffusion (AGID) test results can have viral loads at the same level as animals with strong antibody responses, and are therefore equally likely as a potential source of transmission (Scicluna *et al.*, 2013).

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 (Suzuki *et al.*, 1982) 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 (McConnico *et al.*, 2000; 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 (Toma, 1980). 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 ELISAs. The AGID test is specific, thus has the advantage of distinguishing between EIA and non-EIA antigen–antibody reactions. Discrepancies between testing methods or tests with questionable results can be further evaluated by immunoblot testing (Issel *et al.,* 1999; 2013; Rusvai *et al.*, 2009).

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

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Identification of the agent[a]						
PCR	–	+/–	–	+/–	–	–
Virus isolation	–	–	–	+	–	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
AGID	++	++	++	++	++	–
ELISA	++	++	++	+	+	–
Immunoblot	–	++	++	++	–	–

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]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 onto leukocyte cultures prepared from horses free of infection. Virus production in cultures can be confirmed by detection of specific EIA antigen by ELISA (Shane *et al*., 1984), by immunofluorescence assay (Weiland *et al*., 1982), or by molecular tests. Virus isolation is rarely attempted because of the difficulty of growing horse leukocyte cultures.

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, 2001). 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, 2001; 2007). A real-time reverse-transcriptase PCR assay has also been described (Cook *et al*., 2002). 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 (Cappelli *et al.,* 2011; Quinlivan *et al.,* 2007).

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, before serum antibodies to EIAV develop;

v)   Ensuring that horses that are to be 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.

2. Serological tests

Due to the persistence of EIAV in infected equids, detection of serum antibody to EIAV confirms the diagnosis of EIAV infection.

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 (Malmquist *et al.*, 1973), from a persistently infected canine thymus cell line (Bouillant *et al.*, 1986), or from proteins expressed in bacteria or baculovirus using the recombinant DNA technique (Archambault *et al.*, 1989; Kong *et al.*, 1997). 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.

To obtain a satisfactory antigen from spleen, a horse must be infected with a highly virulent strain of EIAV. The resulting incubation period should be 5–7 days, and the spleen should be collected 9 days after inoculation, when the virus titre is at its peak and before any detectable amount of precipitating antibody is produced. Undiluted spleen pulp is used in the immunodiffusion test as antigen (Coggins *et al.*, 1973). Extraction of antigen from the spleen with a saline solution and concentration with ammonium sulphate does not give as satisfactory an antigen as selection of a spleen with a very high titre of EIA antigen.

Alternatively, 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 (Malmquist *et al.*, 1973). EIAV core proteins, expressed in bacteria or baculovirus, are commercially available and find practical use as high quality antigens for serological diagnosis.

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 (Montelaro *et al.*, 1984). 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 (Zhang *et al.*, 1999).

2.1.2. Preparation of standard antiserum

A known positive antiserum may be collected from a horse previously 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 serum. It is essential to balance the antigen and antibody concentrations in order to ensure the optimal sensitivity of the test. Reagent concentrations should be adjusted to form a narrow precipitation line approximately equidistant between the two wells containing antigen and serum.

2.1.3. Test procedure (Association Française de Normalisation [AFNOR], 2000; Coggins *et al.*, 1973; Pearson & Coggins, 1979)

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 24–48 hours the precipitation reactions are examined over a narrow beam of intense, oblique light and against a black background. 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 tend to be diffuse. Such reactions can be confirmed as specific for EIA by dilution at 1/2 or 1/4 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. 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 2 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.

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. 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. If commercial ELISA materials are not available, a non-competitive ELISA using p26 antigen purified from cell culture material may be employed (Shane *et al.*, 1984).

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 immunoblot technique. A standard antiserum for immunodiffusion, which contains detectable antibody, is available from the WOAH Reference Laboratories[1]. 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).

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

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1   https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

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.

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. 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

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2019.