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Equine Infectious Anaemia

Pathology and Serology

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Contents

Equine Infectious Anaemia

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1. Introduction

Equine infectious anaemia (EIA) is a disease of horses caused by a non-oncogenic retrovirus which is transmitted by biting insects. It may present as an acute disease but is more usually characterised by a long chronic illness, with anaemia, recurrent febrile episodes and emaciation, after an initial acute attack. Inapparent infections may also occur.

The disease has been diagnosed in isolated instances in New South Wales, the Northern Territory and in Victoria, but surveys have shown a low to very low prevalence of serological reactors, or of clinical cases. By contrast the disease is well established in certain areas of Queensland. On present information the endemic areas would appear to be the inland river systems of western Queensland from the south-west to the Gulf and the Dawson–McKenzie River basins of central Queensland. In Victoria the disease, in the main, was restricted to a herd of horses maintained for serum production.

Confirmation of a diagnosis of EIA by transmission test to a susceptible horse is not a practical routine procedure but an accurate diagnosis can be made on clinical signs, haematological changes, gross pathology and histopathology, and the demonstration of serum antibody.

2. Clinical Signs

The chronic disease is characterised by progressive loss of condition, lack of exercise tolerance, muscular weakness, rough coat, depression and anaemia. There may be recurrent febrile attacks associated with fluctuating dependent oedema throughout the course of the chronic disease which usually lasts for 3–12 months. Deaths usually occur following a severe primary acute attack or an acute episode following chronic disease. In the acute stages there is an elevated temperature (up to 41°C), severe depression, anorexia, ataxia, conjunctival congestion and ocular and nasal discharge, jaundice, diarrhoea, and oedema which may involve the head, neck, ventral underline and extend down one or more limbs.

3. Haematology

3.1. Changes in Blood Parameters

The anaemia observed in EIA is a normocytic, normochromic anaemia. Thus, the estimation of haemoglobin, packed cell volume and red cell count are of value. Haemoglobin values as low as 2.0 g/dL have been recorded. The sedimentation rate of the freshly drawn blood is elevated but this has not been found to be sufficiently consistent or marked to be particularly helpful.

Leucocyte values are not significantly abnormal. There is often a mild neutrophilia at the onset of clinical signs, followed by a moderate monocytosis.

3.2. Sideroleucocyte Smears – Preparation and Interpretation

Blood (9 mL) is added to 1 mL 0.34 mol/L trisodium citrate ($Na_3C_6H_5O_7.2H_2O$) and allowed to stand for one hour at room temperature in which time the red cells settle to the bottom of the tube. The supernatant plasma is pipetted off and spun at about 800 *g* for 10 min. There will be a pellet of leucocytes at the bottom of the centrifuge tube. The supernatant plasma is pipetted off and discarded except for two to three drops which are mixed with the leucocyte pellet.

A large drop of leucocyte suspension is placed at one end of a microscope slide and a thick smear prepared by pushing rapidly and lightly with a 22 mm^2 coverslip held at about 45° to the slide. Smears are air dried.

3.2.1. Staining

(a) Fix smears for one minute with methanol.
(b) Air dry.
(c) Stain for 20 min with freshly prepared mixture of equal volumes of 2 mol/L hydrochloric acid (HCl) and 0.24 mol/L potassium ferrocyanide [$K_4Fe(CN)_6.3H_2O$] in distilled water.
(d) Wash lightly with tap water.
(e) Counterstain for 10 min with 0.2% Pyronin.
(f) Wash lightly with tap water.
(g) Blot lightly and air dry.

Smears are examined using an oil immersion objective. Leucocytes stain pink. Sideroleucocytes are identified as leucocytes containing a single blue granule, a diffuse blue deposit or both. The number of sideroleucocytes per 10 000 leucocytes is counted. In a well-prepared smear there are 200–300 leucocytes per field.

A thorough examination of Giemsa stained erythrocytes also should be undertaken to eliminate the possibility of acute babesial infections.

3.2.2. Interpretation

Two or more sideroleucocytes per 10 000 leucocytes are considered highly indicative of EIA. They are found during the febrile stage of EIA, two to three days after the initial temperature rise and may persist for two to three weeks. In some animals there are long periods between febrile reactions when sideroleucocytes are not detected. Thus negative results do not necessarily exclude this disease. Caution in interpreting sideroleucocyte smears may be warranted if iron injections have been given.

4. Gross Pathology

In acute cases there is icterus, subserosal haemorrhages and oedema of subcutaneous tissue, perirenal and sublumbar fat. The liver is considerably enlarged, firm, with rounded edges and with red to dark-brown mottling. The spleen and lymph nodes are generally enlarged and the

lungs may have a brownish colouration due to accumulation of haemosiderin in alveolar macrophages.

5. Histopathology

All organs may show evidence of reticulo-endothelial hyperplasia. Adequate descriptions of the microscopic changes occuring in EIA are contained in standard veterinary pathology texts (e.g. Jones and Hunt, 1983) but the most striking changes occur in the liver and they will be described in some detail here. There is congestion of central veins and dilation of adjacent sinusoids. The sinusoids contain considerable numbers of lymphocytes, plasma cells and macrophages containing haemosiderin or erythrocytes. Kupffer cells are enlarged and are frequently filled with haemosiderin. Perhaps the most characteristic change is the occurrence of nests of proliferating reticuloendothelial cells which may develop at any point along the length of the sinusoid. As these nests expand they cause compression and degeneration of adjacent parenchymal cells. Portal triads and Glisson's capsule are also infiltrated by mononuclear cells.

6. Serological Examination for Equine Infectious Anaemia

The gel diffusion test is a simple and reliable technique for the diagnosis of EIA. The test was first described by Coggins and Norcross (1970), and is often referred to as the Coggins test.

Attempts in Australia to isolate EIA virus in tissue culture using the equine dermal cell line CCL57, available from Commonwealth Serum Laboratories, have been unsuccessful to date. Consequently antigen for use in the gel-diffusion test must be prepared from the spleen from infected horses. The method for this is given below together with details of the test procedure.

A satisfactory alternative is to purchase the imported Pitman–Moore Equine Infectious Anaemia Immunodiffusion Test Kit (antigen of equine cell line origin; reagent serum, equine origin) catalogue reference 55150.20 (Pitman-Moore, Box 344 PO, Washington Crossing, New Jersey, O8560, USA).

In Australia import approval has been granted to Heriot Agencies, 5/16 Macquarie Place, Boronia, Vic. 3155, on the conditions that the kits are used *in vitro* for diagnostic purposes only and that the reagents are treated as though they may be infectious.

The kit test antigen and an Australian antigen preparation in alternate wells have been found to give a single line of identity against Australian and kit test antisera placed in the central well. Further, identical results have been obtained when Australian sera are tested with the kits or with Australian reagents. Detailed instructions on performance and interpretation of the test are provided. Unfortunately, strong and weak positive and negative control sera are not supplied and these should be obtained from a laboratory experienced in performance of the test [e.g. the Animal Research Institute, Yeerongpilly, Qld 4105. Tel. (07) 362 9400; Fax (07) 892 5374].

6.1. Preparation of Antigen

6.1.1. Infection of Horse with Virus

A serologically negative horse is inoculated intravenously with 50–100 mL of plasma or serum collected from an EIA infected horse during a febrile period. A horse with a rectal temperature of 39.5°C or more 6–10 days after inoculation is suitable for harvest of spleen, and as a source of infective material for passaging the virus. Repeated passage may be necessary before a serologically active spleen is obtained.

The horse is killed after two to four days of fever. The spleen is removed and cut into convenient pieces for storage at -20°C prior to extraction.

6.1.2. Extraction of Antigen from Spleen

Spleen-extract antigen is prepared according to the method of Henson *et al.* (1971), as follows.

(a) Mince the thawed spleen with scissors.
(b) Add 1.2 mL of cold phosphate buffered saline (PBS) per gram of minced spleen.
(c) Emulsify in a Waring blender for five minutes in a cold room.
(d) Freeze and thaw the suspension twice.
(e) Centrifuge at 2000 *g* for 10 min at 4°C.
(f) To 2.2 volumes of supernatant, add one volume of cold trichlorotrifluoroethane (CF_3CCl_3).
(g) Emulsify in a Waring blender for five minutes in a cold room.
(h) Centrifuge at 12 000 *g* for 15 min at 4°C.
(i) Precipitate the supernatant with an equal volume of saturated ammonium sulfate [$(NH_4)_2SO_4$]. Collect the precipitate by centrifugation. Resuspend the precipitate in PBS to the original volume before the addition of saturated ammonium sulfate. Precipitate with a quantity of saturated ammonium sulfate equivalent to 90% of an equal volume. The sediment is collected by centrifugation and reconstituted to the original volume with PBS and dialysed against PBS to remove the ammonium sulfate. This may be checked by adding an equal volume of a 0.41 mol/L barium chloride ($BaCl_2.2H_2O$) solution to the PBS. Formation of a white precipitate indicates the presence of sulfate.
(j) Adjust the concentration of the antigen according to its reaction with a range of positive sera including a weak and strong serum in the gel diffusion test.

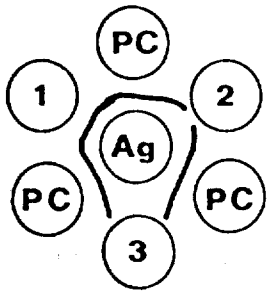

Figure 1. EIA gel diffusion test. Ag = antigen. PC = positive control serum. Serum No. 1 is positive. Serum No. 2 is weakly positive. Serum No. 3 is negative.

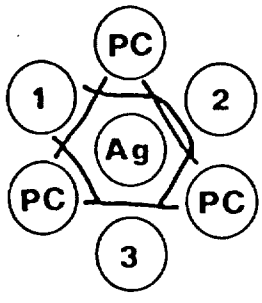

Figure 2. EIA gel diffusion test. Non-specific and specific reactions. Serum No. 1 is negative. Serum No. 2 is positive. Serum No. 3 is unreadable, may be a reaction of partial identity, or a negative or positive; repeat with a different batch of antigen, adjust the concentration of the serum or repeat with a fresh sample.

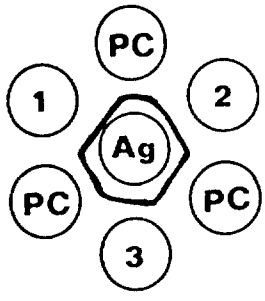

Figure 3. Well No. 1 contains very strong positive serum. Wells 2 and 3 contain 1/2 and 1/4 dilutions of this serum, respectively.

Concentration is preferably achieved by ultrafiltration using an Amicon PM-10 membrane (Amicon Corporation, Lexington, USA) but vacuum dialysis or evaporation are also satisfactory. If ultrafiltration is used dialysis of the ammonium sulfate precipitate against PBS may be omitted.

(k) Preserve the antigen with 0.015 mol/L sodium azide (NaN_3) and store frozen at -20°C. Continual freezing and thawing does not lower the potency of the antigen.

6.2. The Gel Diffusion Test

The test is performed in 100 mm diameter petri dishes into which 15 mL of 1.5 times Noble agar (Difco Laboratories, Detroit, Michigan, USA) in PBS pH 7.2, preserved with 0.015 mol/L sodium azide has been layered.

PBS	pH 7.2
Sodium chloride	8 g
Potassium chloride, KCl	0.2 g
Anhydrous disodium hydrogen phosphate, Na_2HPO_4	1.15 g
Anhydrous potassium dihydrogen phosphate, KH_2PO_4	0.2 g
Distilled water	1 L

A seven-well pattern consisting of a central well and six peripheral wells is cut into the agar. Wells are 6.5 mm in diameter and spaced 2.5 mm apart. The plates of uncut agar may be stored up to five days at 4°C in a moist chamber prior to use.

The central well is filled with antigen (about 0.05 mL), and positive control and test sera are alternated in the six peripheral wells, so that a test serum is always adjacent to two control sera. The control serum is drawn from an infected horse and is selected to give a clear line of precipitation midway between the antigen and serum wells. It is preserved with 0.015 mol/L sodium azide.

The plates are incubated at 37°C in a moist chamber and read daily for up to 72 hours. The wells are not refilled during incubation. Positive tests are usually evident after overnight incubations.

6.3. Reading the Test

During the incubation, antigen and antibody diffuse into the agar and in the case of a specific union between antigen and antibody, form a visible line of precipitation in the region where the optimal concentration ratio of antigen to antibody occurs. The reaction is best observed with oblique light over a black background. A positive result is indicated by a line of precipitation which joins the adjacent reference line in a reaction of identity (Fig. 1).

A weak positive is indicated by curving of the reference line towards the test serum well, in the absence of a line between the test serum and the antigen well (Fig. 1).

In the case of a negative test, the adjacent reference line continues straight on to the test well (Fig. 1). The test is very specific, and reactions of nonidentity as shown in Fig. 2, are rare.

A very strong positive serum may cause a hazy line to form very close to the antigen well, and is recognised by inhibition of the adjacent reference lines. Such a reaction should be checked by diluting the serum 1/2, 1/4 and 1/8 in PBS, and repeating the test (Fig. 3). Results are reported as positive or negative.

6.4. Interpretation

Horses infected with EIA virus develop detectable levels of precipitating antibody 14–40 days after

infection, and usually about 10 days after the first pyrexia. The precipitins persist at least for several years, and probably for life.

A positive result indicates that the horse has, at some time, become infected with EIA virus, and should be regarded as a carrier of the disease. Since maternal antibodies may persist for up to six months, results with young foals need to be interpreted accordingly.

7. References

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Henson, J.B., McGuire, T.C., and Gorham, J.R. (1971). The detection of precipitating antibodies in equine infectious anaemia and partial purification of the antigen. *Archiv für die gesamte Virusforschung* **35**, 385–91

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