

Diagnosis of Equine Infectious Anemia

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Equine infectious anemia (EIA) is an infectious disease of Equidae caused by a virus. This is one of the most serious diseases in Japan and has resulted in severe economic damage to the equine industry.

The diagnostic methods are described in this paper.

Clinical and hematological diagnosis

The terms, acute, subacute, chronic and relapsing types have been used to classify EIA cases. These types have been correlated with clinical and pathological observations. The acute and subacute types have severe clinical symptoms and are often followed by death while the chronic type is identified by the occurrence of intermittent febrile attacks in a surviving case of the disease and with mild clinical symptoms.

1) Fever

Although the degree of fever depends upon the severity of disease, the characteristic fever of this disease is 40 to 41°C or more and the febrile stage usually continues for 4 or 5 days. In the acute and subacute types, the febrile stage continues for a few days longer. There are typical, recurrent or intermittent febrile periods of about 39°C or more in the chronic type of the disease.

2) Anemia

The anemia usually begins at the peak of the febrile stage, becomes quite evident after

the fever crisis, and gradually returns to normal if the fever does not recur. The curve showing the relationship between the fever and anemia is very characteristic of EIA. Severe anemia with a decrease of erythrocyte count to 1 or 1.5 million per mm³ of blood sometimes occurs following a single febrile attack. Anemia, however, is not usually seen in the chronic cases which continue to be afebrile.

3) Appearance of sideroleukocytes in the circulating blood

The sideroleukocytes which appear in the circulating blood of EIA cases are mononuclear cells and neutrophils which contain an iron-positive reacting substance such as ferritin and hemosiderin.

In EIA, when there is recurrence of fever, anemia occurs at the time of the febrile crisis, and sideroleukocytes appear and increase in number in the circulating blood.

The greatest probability of detection of sideroleukocytes is at 1 to 4 days after the fever crisis. The febrile period and during the afebrile stage at 5 to 10 days after the fever crisis are the next period of high probability of detection⁹⁾.

Sideroleukocytes also appear in the circulating blood of horses affected with trypanosomiasis and piroplasmiasis. These diseases, however, can be differentiated from EIA by finding the parasites in the blood smears.

In our experience, very few or no sideroleukocytes have been found in the circulating blood of adult horses infected with diseases

other than EIA, trypanosomiasis and piroplasmiasis. In Japan, where neither piroplasmiasis nor trypanosomiasis is known to occur, horses in which one or more sideroleukocytes are found per 10,000 leukocytes are legally considered to be positive for EIA⁹.

The method⁹ which has been used in Japan for the detection of sideroleukocytes in the blood is carried out as follows:

(1) One milliliter of 10 per cent sodium citrate solution is placed in a test tube.

(2) About 9 ml. of blood is taken from the jugular vein and mixed with the solution.

(3) The tube is allowed to stand at room temperature for 1 to 2 hours, during which time the erythrocytes are sedimented.

(4) The plasma is transferred to a 15 ml. conical centrifugal tube.

(5) The plasma is centrifuged at 1,000 rpm for 3 to 4 minutes.

(6) The resulting supernatant is discarded.

(7) The sediment is taken up with a capillary tube.

(8) A leukocyte smear is prepared from a drop of the sediment and dried in air.

(9) The leukocyte smear is fixed in methyl alcohol for 2 to 3 minutes and dried in air.

(10) The smear is flooded with Prussian blue solution for 10 to 20 minutes and then washed with water for 5 minutes or more.

(11) It is then counter-stained with a 0.1 per cent pyronin solution, washed with water, and dried.

(12) The smear is examined under a microscope using the oil immersion objective. Iron-positive substances stain blue and the nuclei of the leukocyte stain red.

4) *Other changes of the blood and clinical signs*

Although a neutrophilia is observed at the febrile stage, relative lymphocytosis and monocytosis are present after the crisis. The erythrocyte sedimentation rate is also increased. Weakness, loss of appetite, edema and generalized icterus often appear in severe cases.

Cardiac weakness is an important clinical sign because of its presence even in those chronic cases with normal temperature.

Histopathological diagnosis by liver biopsy

A diagnostic method based on the histopathological examination of liver biopsy tissue specimens has been applied to latent and asymptomatic cases of EIA, especially those in which it was difficult to reach a diagnosis by clinical or hematological methods.

The instrument for liver biopsy which has been used in Japan is illustrated in Fig. 1, and the technique for liver biopsy of the horse is as follows:

(1) The needle used for this purpose is 25.3 cm in length and consisted of an outer (A) and inner (B) needles and a cutter (D).

(2) The intercostal space between the 14th and 15th ribs on the right thorax at a point

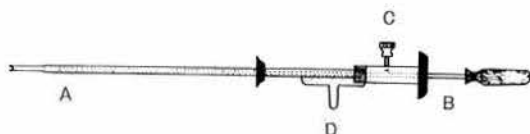


Fig. 1. Instrument for liver biopsy

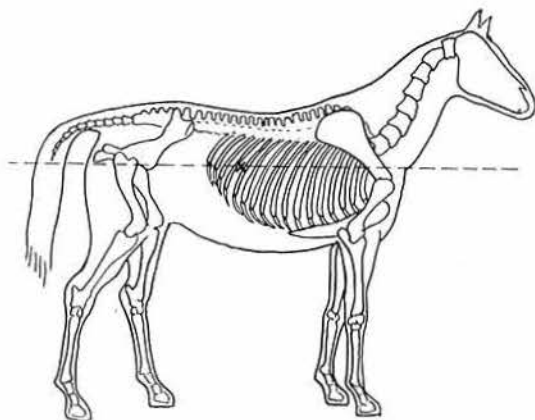


Fig. 2. Site of entry for liver biopsy

through a horizontal line passing through the coxo-femoral joint is selected as the site for entry (Fig. 2).

(3) The site is shaved, disinfected, and a small incision is made in the skin.

(4) The sterilized biopsy needle is introduced through the skin incision and muscles directly into the thoracic cavity.

(5) When the tip of the needle encounters the diaphragm, the needle moves with the movement of the diaphragm. It is then directed to the elbow joint of the opposite side.

(6) The inner needle is then drawn out 5 to 7 cm and fixed with a screw. Make sure that the cutter is pulled back.

(7) The needle is then pushed as far as the stop to penetrate the liver parenchyma, the cutter pushed forward, and then the needle is withdrawn from the animal all in a single smooth motion.

(8) The small piece of liver tissue found within the outer needle is removed by expulsion with the inner needle.

(9) The skin incision is disinfected and sutured.

(10) The liver tissue is immediately fixed in 10 per cent buffered formalin. It is then embedded in paraffin, and 6μ sections are made and stained with hematoxylin and eosin and with Prussian blue.

(11) The liver specimen is examined under a microscope.

In severe cases, there is a severe proliferation and desquamation of Kupffer cells or endothelial cells of the sinusoids, focal proliferation of reticuloendothelial cells and lymphoid cells in the acini and Glisson's sheath, and degeneration of liver cells.

In mild EIA, a slight swelling and desquamation of Kupffer cells or sinusoidal endothelial cells occurs in the acini, and nodular and diffuse accumulation of lymphoid cells occurs in Glisson's sheath.

There are, however, only minute lesions involving a few round cells in the acini and Glisson's sheath of the liver in these EIA cases diagnosed on the basis of lesions of the

spleen and lymph nodes. In these cases, hepatic lesions are not characteristic of EIA⁹.

Diagnosis using the horse inoculation test

For the EIA diagnosis, the horse inoculation test is still the most important method, which, however, is not practical from the standpoint of economy. The conduct of the test which has been used in our laboratory is as follows:

Two healthy horses 6 to 18 months of age which have been observed clinico-hematologically for 2 months are inoculated subcutaneously with 100 ml of blood from the suspected horses. They are then observed clinico-hematologically for 3 months after inoculation.

When one or both animals are infected with EIA, the suspected horse is considered to be EIA, and when both animals are not infected, the suspect is considered to be free of EIA.

Diagnosis using the horse leukocyte culture method

The cultivation of the EIA virus in horse leukocyte and bone marrow cell culture was first accomplished by Kobayashi⁹. The cell culture technique employs equine leukocytes obtained from heparinized blood aseptically withdrawn from the jugular vein of healthy horses.

The blood is allowed to stand at room temperature for about 60 minutes or until the erythrocytes are sedimented. The plasma layer is collected and centrifuged at 1,000 to 1,500 rpm for 7 minutes.

The leukocyte sediment is washed once in Hanks solution, suspended in nutrient medium at a concentration of approximately 20 million cells per ml and incubated as a stationary culture at 37°C.

Modified Carrel flasks, bottles and tubes which contain 2, 10 and 0.5 ml of cell suspension, respectively, have been used. The

tissue culture media used is either whole bovine serum or a mixture of equal volumes of bovine serum and Eagle's medium⁹.

The virus material is inoculated in these cultures on the second day after the beginning of cultivation of the leukocytes. Cultures are examined daily under a microscope.

Seven to 18 days after inoculation of the virus, cytopathic changes shown by clumping, degeneration and desquamation of cells appear.

In doubtful cases, cytopathic changes will definitely appear after a secondary passage of the cultured fluid into a new horse leukocyte culture if the virus is present. The complement fixation test is also used to detect the growth of virus in the tissue cultures.

The leukocyte culture technique has not yet been perfected to the point where it might serve as a substitute for the horse inoculation test. Some problems remain, particularly those pertaining to the maintenance of a constant leukocyte culture.

An important factor is the selection of a suitable leukocyte donor by the testing of several horses and to obtain an optimum combination of horse leukocytes and of bovine serum for use in the leukocyte culture.

Diagnosis using serological tests

The complement fixation (CF) test is used for the EIA diagnosis^{7,8}. The virus material is prepared from a leukocyte culture of the EIA virus. After one cycle of freezing and thawing, the culture is centrifuged at 3,000 rpm for 15 minutes and the resulting supernatant solution is used as the antigen.

As for the application of the CF test for the diagnosis of EIA in the field, it is of much less value because the CF antibody against EIA remains elevated for only a short period of time and it does not show a second rise after its disappearance. It can, however, be of practical value for diagnosis when the test is used in mass outbreaks which occur within a relatively short period of time.

Virus neutralizing antibody has been de-

monstrated in the sera of EIA infected horses and the practical application of the virus neutralization test for diagnostic purpose is currently under investigation in Japan⁹.

Recently, an agar-gel immunodiffusion test was developed by Coggins & Norcross⁹ in the United States. In this test, the antigen was prepared from the spleen of an infected horse at the acute stage of infection with EIA by the process of freezing and thawing and by chopping.

Another agar-gel immunodiffusion test using purified EIA virus as antigen was developed by Nakajima & Ushimi¹⁰ in Japan. It seems that this test may be very highly specific for EIA, and is considered to be hopeful, because the antibody demonstrated by this test is group-specific for EIA virus and remained in the blood for a long time.

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