

Diagnosis of Equine Infectious Anemia by Immunodiffusion Test

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Equine infectious anemia (EIA) is a viral disease of equidae characterized by a lifelong persistent infection, intermittent fever, anemia and progressive weakness. The virus is spherical particles approximately 100 m μ in diameter which contain RNA. Histopathologically, proliferative lymphoid cells are generally observed in the reticular endothelial system.

Until recently, EIA was extremely difficult to diagnose and many attempts had been made to develop a reliable diagnostic test. Of them, complement fixation and neutralization tests using the horse leukocyte culture technique have been shown to be specific for EIA and they will detect EIA under optimal conditions.

However, complement-fixing (CF) antibody is usually detectable for only a short period after the first febrile reaction, although it reacts with immunologically different strains of EIA virus which are distinguishable from one another by neutralization test.

Neutralizing antibody, on the other hand, persists for a long period of time but does not react with antigenically different virus strains. Accordingly, both tests are not entirely satisfactory for diagnostic use.

Recently, Coggins and Norcross⁴⁾, and Nakajima and Ushimi⁹⁾ developed immunodiffusion tests for EIA using infected horse spleen and infected horse leukocyte cultures as antigen, respectively.

The immunodiffusion test detects EIA-virus-specific precipitating antibody^{9),12)} which appears early in the infection, remains for a long period in the serum, and can be demonstrated

in all infected horses^{4),5),7),11)}, and even in horses infected with immunologically distinct virus strains^{9),11)}. Based on these findings, the immunodiffusion test appears to be a reliable and practical means for the diagnosis of EIA^{3),4),7),13),14),19)}.

Procedures for immunodiffusion test

1) General procedures

One per cent solution of purified agar is prepared in 0.01 M phosphate buffer (pH 7.4) containing 0.85 per cent NaCl and 0.01 per cent thimerosal. Thimerosal will inhibit growth of bacteria and fungi. The agar is dissolved by boiling for 10 minutes. Then, 4.5 ml of the agar solution is poured on microscope slides (26 × 76 mm). The plate is allowed to harden.

A seven-well puncher with one central and six peripheral wells are used for cutting wells in the agar. Wells are 5 mm in diameter and peripheral wells are placed at a distance of 3 mm from the central well. The puncher for this purpose can be purchased. Three sets of these combined wells can be prepared on one plate¹⁴⁾.

Antigen is filled in the central well and reference positive serum in the left and right peripheral wells as shown in Fig. 1. Test sera are sampled in the other four peripheral wells. Accordingly, 12 serum samples can be tested using one plate. Approximately 0.05 ml of sera or antigen is needed for one well.

The plate is incubated in a moist chamber

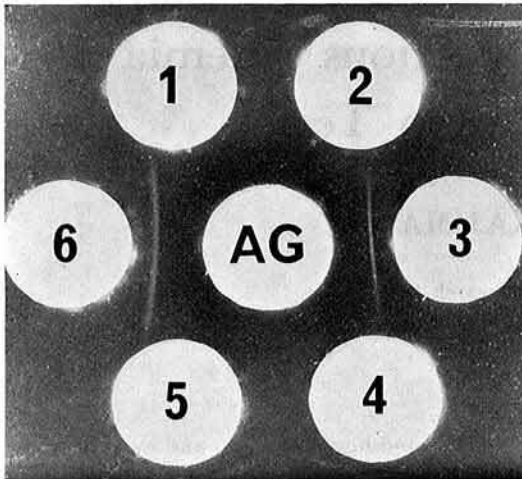


Fig. 1. Control precipitation lines in the immunodiffusion test. Antigen (AG) is placed in the central well and control positive serum in wells 3 and 6. When reaction is allowed to occur, a control precipitation line is always produced between antigen and each control positive serum. Serum samples to be tested are placed in wells 1, 2, 4, and 5 and allowed to react with antigen

for three days after sampling. Although the control precipitation lines can be seen within 24 hours, weak reactions are not visible at 24 hours. When the reaction is completed, the plate is photographed unstained for accurate record. If it is difficult to get photographs, accurate drawing is necessary. An example of recording paper is presented in Fig. 2.

When it is necessary to preserve the plate, it is washed for three days in saline to remove the substance which is not involved in the reaction and then rinsed for a few hours in distilled water to remove sodium chloride.

Finally, the plate is dried, stained with amido black, destained and preserved. Staining solution is prepared by mixing 0.1 gram of amido black 10 B, 70 ml of methyl alcohol, 10 ml of acetic acid and 20 ml of distilled water.

2) Preparation of immunodiffusion antigen

Purified EIA virus was first used as antigen⁹⁾. For field application of the immunodiffusion test, however, it is important to

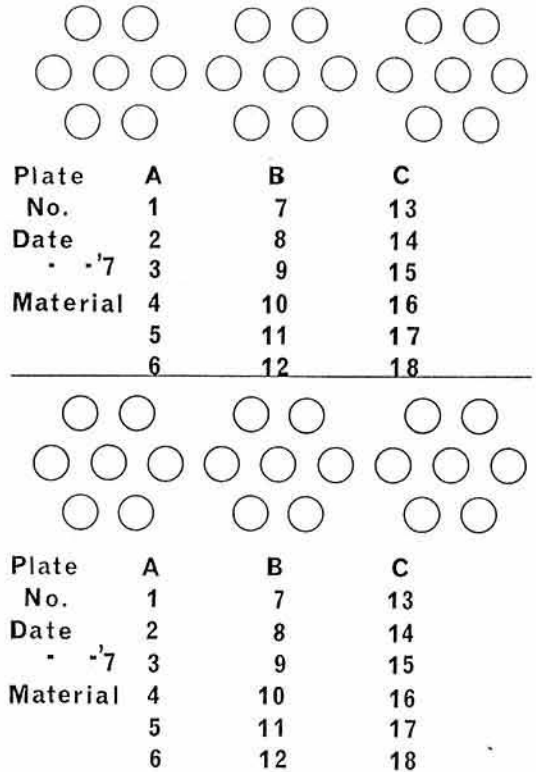


Fig. 2. An example of recording paper

develop simple procedures for preparation of large amounts of EIA-specific antigen.

At present, antigen is prepared by collecting cultured horse leukocytes as well as EIA virus by ultracentrifugation in a small amount of phosphate-buffered saline containing 0.1 per cent Tween 80 and by treating them with two volumes of ether twice¹⁵⁾. These procedures are described in Fig. 3. One of the examples for antigen preparation is shown in Table 1.

Since this antigen has been proven to be antigenically identical with purified EIA virus and can be obtained by simple procedures, the antigen seems to be a suitable one for the EIA field survey. Antigen employed in many countries of the world is prepared mostly from infected horse spleen^{1), 2), 5-7), 15)}, except in two laboratories^{8), 15)}.

There is an optimal antigen-antibody ratio in immunodiffusion reaction. If the activity of antigen or antibody is highly unbalanced, precipitation line does not appear or appears

Horse leukocyte culture	Suspend horse leukocytes in 100 % bovine serum at a rate of $2 \cdot 4 \times 10^7$ cells per ml and incubate at 37°C for 20 hours.
Inoculation	Remove medium, inoculate seed virus and add 100 % bovine serum. Incubate at 37°C for 24 hours.
Medium replacement	Replace with 1:1 mixture of ultracentrifuged bovine serum and Eagle's medium. Incubate at 37°C.
Collection of infected cultures	Collect infected culture fluid and cells when CPE appeared.
Precipitation of virus antigen	Precipitate antigenic substance from infected cultures by ultracentrifugation at $100,000 \times g$ for 120 minutes.
Extraction of antigen	Suspend the precipitate in PBS containing 0.1 % Tween 80, treat with ether and centrifuge at $1,000 \times g$ for 20 minutes. Collect fluid phase.
Group-specific antigen of EIA virus	

Fig. 3. Procedures for immunodiffusion antigen preparation

weakly. Therefore, optimal antigen concentration has been determined against various concentration of antibody by box titration¹⁶⁾. Results indicate that antigen with a titer of 1:8 or 1:16 is satisfactory and adequate against serum samples having a titer ranging from 1:1 to 1:128. Based on the result, antigenic titer is adjusted to 1:8 before use.

The antigen produced in the experiment of Table 1 has a titer of 1:32. This can be diluted fourfold for diagnostic use. Accordingly, 80 ml of immunodiffusion antigen was prepared from a single lot of horse leukocyte cultures. With the antigen thus prepared, more than 6,000 horses could be tested by immunodiffusion test for EIA diagnosis.

3) Preparation of control positive serum

Control positive serum is obtained from a horse experimentally infected with EIA virus. Optimal precipitating antibody titer as positive control serum has been determined to be 1:4 by box titration¹⁶⁾. Therefore, titer of the

Table 1. One example of antigen preparation

Horse blood	Infected culture fluid and cells	Extracted antigen	Antigenic activity
3,000 ml	500 ml	20 ml	1 : 32

serum is adjusted to become 1:4 before use. Then, it is confirmed to match with purified EIA virus and to form only one distinct precipitation line.

4) Reading the immunodiffusion test

Control precipitation lines always appear between antigen and each control positive serum, slightly toward the wells of the positive serum (Fig. 1). The control lines are the basis for reading the test. If the distinct control line is not formed, the test must be repeated.

The type of reaction varies with the strength of antibody titer in the test serum. When the control line joins with and forms a continuous line with the line between the test serum and antigen, the sample is regarded as positive one.

When the control line bends slightly toward the inside of the well of the test serum but does not form a complete line, or the control line bends and hits inside of the well of the test serum, this sample is regarded as suspectedly positive one.

When the control line continues into the test serum well without bending or bends toward the outside of the well as presented in Fig. 1, the test serum is regarded as negative one. Occasionally, double precipitation lines are observed. Their significance has not been determined.

A precipitation line formed, sometimes, does not join the control line smoothly, or cross the control line. This is due to the reaction other than EIA virus antigen and its antibody. These reactions are regarded as non specific ones. Suspectedly positive cases should be tested again two to three weeks later. The reaction probably becomes stronger and is easy to read.

Characteristics of immunodiffusion antigen

1) Antigenic substance

Purified EIA virus has antigenic activity in immunodiffusion reaction⁹⁾, but intact virus has no antigenicity¹⁰⁾. When the virus is disrupted with ether, antigenic activity is demonstrated¹¹⁾. When the antigen is reacted against serum samples from experimental horses infected with antigenically distinct strains of EIA virus which are distinguishable from one another in neutralization test, precipitation lines formed are connected with one another, indicating reactions of identity⁹⁾.

These findings indicate that the antigen involved in the immunodiffusion reaction is a group-specific component of EIA virus which is derived from inner structures rather than surface ones of the virus.

The antigen is a small protein with a molecular weight of 27,500, sedimentation coefficient of 2.1 S, density of 1.18, and isoelectric point of 5.8¹⁷⁾. This antigen is quite heat sensitive and it is inactivated at 56°C for 30 minutes.

2) Antigenic relationship between EIA virus and infected organ derived antigen

Immunodiffusion reaction in EIA was carried out by two separate research groups independently, using infected horse spleen and infected horse leukocyte cultures as antigen, respectively^{4), 9)}. Recently, relationship of these two antigens was determined¹²⁾. Results indicate that antigenic substance derived from EIA-infected horse spleen is identical with that of EIA virus and is a component of the virus. Accordingly, each reaction is detecting the same precipitating antibody.

Immunodiffusion antigen has been demonstrated not only in the spleen but in the liver, lung, kidney, thymus, lymph nodes, bone marrow and serum from infected horses^{15), 20)}. Recently, it is found that cultured fluids from persistently infected equine dermal cells are

one of the most practicable sources of immunodiffusion antigen for quantity production purpose⁸⁾.

Characterization of precipitating antibody

1) Appearance and development of precipitating antibody

Precipitating antibody has been detected in all 18 horses experimentally infected with immunologically distinct various strains of EIA virus. Antibody is detected sometime from 2 to 10 days after the peak of the first pyrexia¹¹⁾ and it seems to remain positive for life.

Antibody titer generally increases by recurrent fever. Its level is, however, considerably variable during the course of disease. Especially, after the horse passed a few months without pyrexia, titer seems to decrease remarkably¹⁶⁾.

Serum samples collected from horses infected with equine influenza virus, equine rhinopneumonitis virus, equine arteritis virus, Japanese encephalitis virus and cytopathogenic equine orphan virus (herpes-type virus) contain, of course, no precipitating antibody specific for EIA.

2) Titer of precipitating antibody

Antibody titer in serum samples from 115 horses positive for both precipitating antibody and sideroleukocytes, and in 56 serum samples from horses positive for the antibody but negative for sideroleukocytes has been examined¹⁶⁾. Maximum titer among them was 1:128. More than 60 per cent of titers were between 1:4 and 1:16 as shown in Fig. 4.

Maximum antibody titer in approximately 200 serum samples taken periodically from 9 horses experimentally infected with EIA virus was also 1:128. Therefore, the titer of 1:128 seems to be the upper limit for precipitating antibody in Japan under the procedure performed in our laboratory¹⁶⁾.

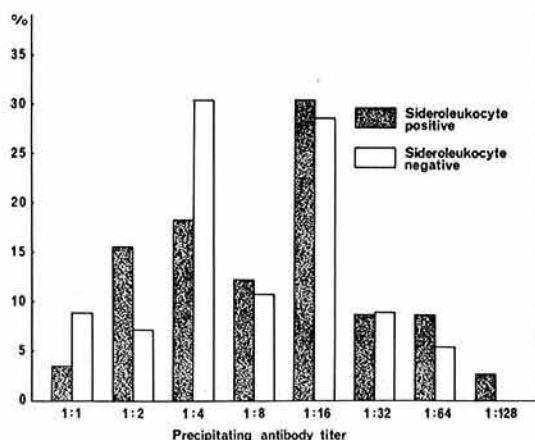


Fig. 4. Distribution of precipitating antibody titer of serum samples collected from field cases with equine infectious anemia. Diagnosis positive for sideroleukocytes is made when one or more sideroleukocytes are detected from among 10,000 leukocytes observed in a smear sample of these cells

3) Relationship to complement-fixing and neutralizing antibodies

Development of precipitating, CF and neutralizing antibodies is sequentially determined in one infected horse as presented in Fig. 5. Results indicate that precipitating antibody appears shortly after the first pyrexia and its rise coincides fairly well with that of CF antibody. Neutralizing antibody is not detectable at this moment.

The CF antibody usually becomes undetectable in a relatively short period but precipitating antibody persists for a long period of time. Neutralizing antibody also remains for a long period. It is, therefore, evident that these three antibodies are different from one another in their development and persistence¹⁰.

4) Physicochemical properties

Precipitating antibody has been detectable in 7S fraction but not in 19S fraction of serum samples obtained in both early and late stages of infection¹⁰. Antibody is shown to be present in gamma globulin (IgG) fraction¹⁰.

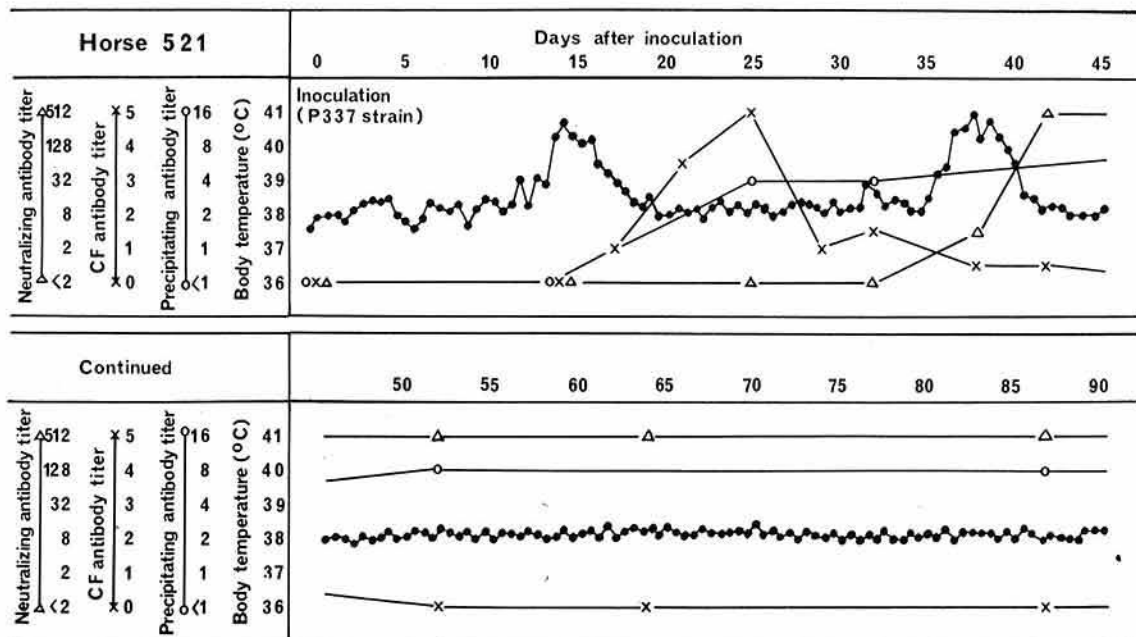


Fig. 5. Responses of a horse to equine infectious anemia virus infection are sequentially measured by precipitating (○—○), CF (x—x) and neutralizing (△—△) antibody titers, and by body temperature (●—●). Antibody titers are expressed as the reciprocal of the highest dilution

Table 2. Field survey of equine infectious anemia by immunodiffusion test

	No. of serum samples tested	Positive	Suspectedly positive	Negative	Percentage of positive cases
Sideroleukocyte positive	152	129	2	21	84.8
Sideroleukocyte negative	8,101	51	7	8,043	0.6

Diagnosis positive for sideroleukocytes is made in the same manner as described in Fig. 4

Field survey EIA by immunodiffusion test

1) Result of field survey

Serum samples from 8,253 horses have been tested. Of them, 152 samples are from horses diagnosed as EIA by the detection of sideroleukocytes in the blood from the jugular vein, and the other 8,101 samples are regarded as non-EIA clinically and hematologically. Results are presented in Table 2.

Precipitating antibody was demonstrated in 0.6 per cent of the sera obtained from horses which appeared to be normal. On the other hand, antibody was detected at a high rate in serum samples from horses positive for sideroleukocytes.

2) Results examined by complement fixation test, pathological findings and sideroleukocyte detection in horses positive for precipitating antibody

Ninety-six horses positive for precipitating antibody have been sequentially examined pathologically by complement fixation test and by sideroleukocyte detection^(9,10,21). There were only 19 cases (20 per cent) positive for CF antibody since the antibody remained positive for relatively a short period after the first pyrexia.

However, approximately 90 per cent of the horses were diagnosed as EIA both pathologically and hematologically. It is obvious that there is a high coincidence in results between the immunodiffusion test and other tests for EIA diagnosis.

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