Diagnosis of Equine Infectious Anemia by Enzyme-Linked Immunosorbent Assay with Viral Antigen Purified by Affinity Chromatography

Takeo Sugiura,* Tomio Matsumura* and Yoshio Fukunaga*

Enzyme-linked immunosorbent assay (ELISA) for the diagnosis of equine infectious anemia (EIA) was developed by using antigen purified by affinity chromatography. The antigen was prepared from the virus which was purified by discontinuous sucrose density gradient ultracentrifugation performed two times. After disrupting the virus with ethyl ether, the antigen was passed through a column in which gel bound with antibody to bovine serum had been packed to remove bovine serum components contained in culture medium.

The plate for ELISA was coated with 0.05 ml of antigen and blocked with gelatin. After washing, 0.05 ml of each undiluted serum sample was added and a reaction allowed to take place at 37°C for 90 min under continuous agitation. All the plate reacted with 0.025 ml of anti-horse IgG conjugated with horseradish peroxidase for 60 min under the same condition as used after washing. Then, 0.25 ml of 0.08% of ABTS and 0.003% hydrogen peroxide were added. A reaction was allowed to take place at room temperature for 50 min. After that, 0.05 ml of salicylic acid was added.

Optical density (OD) ranged from 0.84 to 2.06 (M= 1.61±0.37) in 18 serum samples which were positive for the agar-gel immunodiffusion (AGID) test. OD ranged from 0.01 to 0.32 (M=0.15±0.06) in 189 negative cases. It ranged from 0.04 to 0.33 (M=0.14±0.08) in 41 negative cases showing non-specific reactivity in the AGID test.

From these results, it was apparent that ELISA was suitable for the diagnosis of EIA when it was performed with antigen purified by affinity chromatography using anti-bovine serum.

Key word: diagnosis, equine infectious anemia, enzyme-linked immunosorbent assay, affinity chromatography

Introduction

In Japan, there was an amendment in the Law of the Prevention of Infectious Diseases of Domestic Animals for diagnosis of equine infectious anemia (EIA) in 1978. As a result, the official diagnostic method was changed from the detection of sideroleukocytes in peripheral blood to that of antibody to group specific (gs) antigen of EIA virus (EIAV) by the agar-gel immunodiffusion (AGID) test. After this amendment, the number of horses regarded to have been exposed decreased rapidly except for the first two years. In the recent two years, 1984 and 1985, there were no reports of the disease.
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The race horses must be examined for ETA once a year and all the exposed horses condemned by law. On the other hand, all the horses that are maintained in the facilities of the Japan Racing Association (JRA) are examined for ETA twice a year. Furthermore, every horse is quarantined at the time of entrance into facilities of JRA. For the quarantine, it is kept in an exclusive stable for two days, because it takes more than 24 h for judgment of the AGID test for ETA. This step interferes practically with training for coming racing.

The enzyme-linked immunosorbent assay (ELISA) has an advantage for its rapidity, high sensitivity and specificity. So, it is used by some groups of workers1-3) to detect antibodies to several diseases, including EIA. In order to apply ELISA to the diagnosis of EIA as a self-defence measure in the facilities of the JRA, the authors developed antigen of EIAV purified by affinity chromatography, in which the bovine serum components in the antigen preparation were absorbed to reduce the non-specific reactivity. This report deals with successful experimental application of ELISA to the diagnosis of EIA.

**Materials and Methods**

**Preparation of antigen.** Co-culture of an equine dermal cell line which was persistently infected with the Wyoming strain of EIAV4) and fetal horse kidney cells was used as a virus source. Supernates of infected culture fluid harvested every week were centrifuged at 36 700 x g for 3 h in an RP 19 rotor (Hitachi Koki Co., Ltd., Katsuta, Japan). The resulting sediment was suspended in 75 m mol/l Tris-HCl buffered saline (pH 7.4) containing 1 m mol/l EDTA (TEN). Then virus was banded two times to discontinuous sucrose-density gradient ultracentrifugation with 20, 30 and 50% (w/w) sucrose in TEN buffer. The banded virus on the 50% cushion was used as an antigen source.

The virus was disrupted by several methods to expose the gs antigen. The ethyl ether treatment which was conducted two times by the method detailed by Nakajima and Ushimi.5) Sucrose-acetone treatment was performed by the method described by Clark and Casals.6) The purified virus was also disrupted by detergents.7) The virus solution was diluted with an equal volume of 2% Nonidet P40 and sodium deoxycholate in 75 m mol/l Tris-HCl buffered saline (pH 7.4). Then, the mixture was incubated at 37°C for 1 h. Finally, antigen was passed through the affinity chromatography column.

**Affinity chromatography.** An IgG-rich fraction was purified by sedimentation with 30% saturated ammonium sulfate from the serum of a rabbit immunized by repeated injection with bovine fetal whole-serum. It was bound to Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) by using cyanogen bromide at 3mg/ml of gel.8) An antigen solution which was dialyzed to 0.1 mol/l Tris-HCl buffer (pH 8.0) containing 1 mol/l sodium chloride was passed through this column made from 50 ml of gel at a flow rate of 40 ml/h at room temperature.

**AGID test.** The AGID test for detection of antibody against EIAV gs antigen was conducted by the method described by Nakajima and Ushimi9) with commercial antigen and standard serum (EIA Diagnostic Antigen, Nisseiken Co., Ltd., Ome, Japan).

Quantitation of antigen and precipitating
antibody was performed also by the AGID test with the same antigen and standard serum at two and three-fold serial dilution. The titer of standard antigen and serum was 8 units by this method.

**ELISA.** Purified antigen which had been dialyzed to 50 m mol/l carbonate buffer (pH 9.6) was placed at 0.05ml amount in wells of an Immulon II ELISA plate (Nunc, Roskilde, Denmark). The plate was dried completely in a lyophilizing chamber at room temperature for about 3 h. The surfaces of the wells were covered with 0.2 ml of 1% gelatin (EIA Purity Reagent, Bio Lad Laboratories, Richmond, California, U.S.A.) dissolved in the same buffer at room temperature for 30 min. Subsequently, they were washed with phosphate buffered saline containing 0.05% Tween 20 and 0.02% sodium azide (PBS-T).

Serum samples which had not been diluted with any solutions were added at 0.05ml amounts to two wells, of which one was antigen-coated and the other not coated. After incubation at 37°C for 90 min under sealing and continuous agitating, the plates were washed. To them was added 0.025 ml of goat anti-horse IgG (H+L) conjugated with horseradish peroxidase (Cappel Laboratories, Inc., Cochranville, Pennsylvania, U.S.A.) which had been diluted 1: 300 with PBS-T in each well.

The plates were sealed and incubated at 37°C for 60 min, and washed 5 times with PBS-T. Then 0.25ml of each substrate was added. The substrates were made from 0.08% of 2, 2'-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and 0.003% hydrogen peroxide in 0.1mol/l citrate buffer (pH 4.0). Each plate was kept in a dark room at room temperature for 50 min. To it was added 0.05ml of 1% salicylic acid to stop the color intensity.

The optical density (OD) at 415nm was measured by the ETY-96 ELISA Reader (Toyo Sokki Co., Ltd., Tokyo). The OD of each sample was expressed as the difference between the OD at the antigen-coated well and that at uncoated well.

**Serum samples.** Serum samples positive
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Fig. 2. Effect of antigen concentration used for coating the plate wells on optical density (OD).

Wells are coated with 0.025 ml amounts of antigen at different concentrations. They react with EIA-infected horse serum having 8 units of precipitating antibody.

Fig. 3. Effect of antibody concentration on optical density (OD).

Wells are coated with 0.5 unit of antigen in the agar-gel immunodiffusion (AGID) test. They react with serial dilutions of infected horse serum showing an antibody titer of 2 units in the AGID test.

for the AGID test were collected from 18 field cases. They were stored in the authors' laboratory to use as antibody-positive sera.

Serum samples negative for the AGID test were collected from 189 horses which were maintained at the two Training Centers of the JRA in 1984. They were used as antibody negative sera. On the other hand, 41 of the non-specific reactors were negative for the AGID test, but showed a precipitating line crossing with the specific reference line, as is clear in Fig. 1. Generally, they presented a rate of 1 to 5% in every examination of EIA at the Training Centers. Serum samples were collected from them.

Results

Optimal antigenic titer for ELISA.

As shown in Fig. 2, OD was essentially in proportion to antigenic titer. Maximum OD was observed at 0.5 unit of antigen. From this result, the optimal antigenic titer was determined to be 0.5 unit.

As shown in Fig. 3, OD was essentially in proportion to antibody titer when 0.5 unit of antigen was coated to the plate. From the repeated examination, the minimum precipitating antibody titer of this test was 0.03 when the OD more than 0.5 is considered to be positive.

Comparison of methods for disrupting the virus. As shown in Table 1, after treatment with ether or detergents, OD was a little higher than after sucrose-ace tone treatment.

Absorption of bovine serum components in antigen by affinity chromatography. To reduce the bovine serum components contained in antigen, the antigen solution was passed through a column packed with gel which was bound with antibody against bovine whole serum. As shown in Table 2, this absorption reduced markedly OD of a serum sample which was negative to EIAV but positive to bovine
Table 1. Effect of 3 methods for disrupting the virus on optical density for enzyme-linked immunosorbent assay (ELISA)

<table>
<thead>
<tr>
<th>Method for disrupting the virus</th>
<th>Optical density at 415 nm</th>
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<tr>
<td></td>
<td>Specific*</td>
</tr>
<tr>
<td>Ethyl ether</td>
<td>1.62</td>
</tr>
<tr>
<td>Sucrose-acetone</td>
<td>1.46</td>
</tr>
<tr>
<td>Detergents***</td>
<td>1.65</td>
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Antigen was adjusted to 0.5 unit in titer by the agar-gel immunodiffusion (AGID) test. It reacted with a serum sample which was positive and had 8 units of antibody in the AGID test(*), or with a serum sample which was negative and showed a non-specific precipitating line in the AGID test(**). ***: Sodium deoxycholate and Nonidet P40 were used at a final concentration of 1%. All the data are expressed with a mean value of 3 wells.

Table 2. Effect of affinity chromatography to absorb bovine serum components contained in antigen preparation on optical density

<table>
<thead>
<tr>
<th>Absorption</th>
<th>Optical density at 415 nm</th>
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<tbody>
<tr>
<td></td>
<td>Specific*</td>
</tr>
<tr>
<td>Before</td>
<td>1.62</td>
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<tr>
<td>After</td>
<td>1.53</td>
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* & **: See the footnote of the Table 1. All the data are expressed with a mean value of 3 wells.

Application of ELISA to serum samples. The test using antigen made by ethyl ether treatment and absorbed by affinity chromatography was applied to field serum samples. The results obtained are shown in Fig. 4. In 18 antibody-positive cases OD ranged from 0.84 to 2.06 (M=1.61, S.D.=0.37). In 189 negative cases OD ranged from 0.01 to 0.32 (M=0.15, S.D.=0.06). In 41 cases of non-specific reaction, OD ranged from 0.04 to 0.33 (M=0.14, S.D.=0.08).

Discussion

In the experiment which was performed to determine an optimal antigen titer, more than 0.5 unit of antigen made it possible to detect every AGID-positive serum. From the results, the sensitivity of this test was estimated to be 33 times as high as that of the AGID test.

To reduce the non-specific reaction, affinity chromatography with antibody to bovine whole serum was used in this experiment. It was considered that a non-specific reaction in the AGID test might often be induced among antibodies which were made by repeated injection of drugs pre-
pared from some bovine organ and bovine serum components in vaccine. Most of the non-specific precipitating lines, shown in Fig. 1, could be eliminated routinely by absorption with bovine serum. By this method, OD was markedly reduced in the serum of a horse having antibody to bovine serum. The authors made an attempt previously to reduce the non-specific reactivity by affinity chromatography. They carried out the indirect hemagglutination test of EIA with an affinity column which had been bound with anti-gs antibody taken from an EIA-infected horse. This method had, however, a possibility of causing non-specific reaction by unknown antibody in horse serum. It was concluded that the method used in the present study was more effective than the conventional method to reduce the non-specific reactivity.

Up to this time several reports have been published on the detection of antibody against EIAV gs antigen. In one of them, Suzuki et al. used antigen made by the method which had been employed to produce commercial antigen for the AGID test. They showed, however, no results obtained from the AGID-negative cases in the field. They mentioned in another presentation that there were several percent of reactors among AGID-negative cases in the field.

On the other hand, Shen, et al. reported an ELISA with p26 gs antigen which had been purified by gel filtration. They obtained almost the same level of OD as the present authors; that is, 0.90 to 1.70 for AGID-positive sera, 0.40 to 0.90 for weakly positive sera, and 0.10 to 0.30 for negative sera. Although there is a difference in the criteria of judgment of the AGID test between Japan and the U.S.A., these data indicated that the ELISA was a useful means for the detection of antibody to EIAV.

ELISA is of high sensitivity and can be readily performed especially on a large number of samples with results obtained within 4 to 5 h. It is expected that ELISA will be a predominant method for the diagnosis of EIA in the field in near future.

Acknowledgments

The authors wish to thank Miss. T. Tsunobonya, Miss A. Sakata, Mrs. S. Shibata and Mrs. Y. Niregi for their technical assistance.

Literature Cited

9) Imagawa, H. and Y. Akiyama. 1978. Elimina-
フィニティークロマトグラフィー精製抗原を使用したエイサ法による馬伝染性貧血の診断：杉浦健夫*、松村高夫*、福永昌夫*（*日本中央競馬会競走馬総合研究所新木支所）——フィニティークロマトグラフィー精製抗原を使用したエイサ法により、馬伝染性貧血（伝貧）の診断を行なうことを試みた。使用した抗原は、不連続粘度密度勾配遠心を2回行なって精製した伝貧ウイルスをエテル処理により破壊し、抗牛全血清抗体を使用したフィニティークロマトグラフィーにより混入する筋繊維細胞液由来の牛血清成分を除去して精製した。エイサ法は、0.05 ml の抗原を経過したエイサ用プレートをゼラチンでブロックし洗浄後、0.05 ml の被検血清を希釈しないで加えて37℃で90分間攪拌しながら反応させ、再び洗浄後ペルオキシダーゼ標識抗馬IgG（H+L）を0.025 ml 加え同様60分間反応させた。次いで、さらに洗浄後0.25 ml の0.08％ 2, 2'-アジノーピス（3-ビルベリンゾチアゾリン-6-スルホン酸）および0.003％過酸化水素を加え同様50分間反応させ、0.05 ml の1％塩化マグネシウムで発色を停止し、415 nm の波長で吸光度を測定した。以上の結果、非特異反応を顕著に減少し、野外から収集した血清の吸光度は、寒天ゲル内沈降反応陽性の18例の血清で0.84から2.06（平均=1.61，標準偏差=0.373），陰性の189例では0.01から0.32（平均=0.15，標準偏差=0.06），陰性ではあるが非特異反応の認められた41例では0.4から0.33（平均=0.14，標準偏差=0.08）であった。以上のことから、本反応は、伝貧の診断に応用可能であることが明らかとなった。