Evaluation of Enzyme-Linked Immunosorbent Assay for Titration of Antibody to Equine Herpesvirus Type 1

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Optical density (OD450) of an indirect enzyme-linked immunosorbent assay (ELISA) for detection of antibody to equine herpesvirus type 1 (EHV-1) was compared with agar gel immunodiffusion test (AGID) which is practically used for titration of antibody to the virus in clinics, complement fixation (CF) and neutralizing antibody titers. The correlation coefficient among them was 0.83, 0.67 and 0.44, respectively. The OD450 of ELISA was strongly correlated with antibody levels which were expressed by AGID as follows. The mean OD450 value ± standard deviation of −, +, ++ and +++ samples in the AGID, was 0.27 ± 0.12, 0.43 ± 0.22, 1.10 ± 0.34 and 1.38 ± 0.26, respectively. Change of OD450 in a horse experimentally inoculated with EHV-1 was the same with that of precipitating antibody titrated with AGID and CF antibody. From these results, it was suggested that ELISA can be practically used in place of AGID for control of EHV-1 infection through titration of antibody to the virus in the field. The antigen for ELISA and AGID was suggested to be as VP 19, an inner component of the virus, because the major peptide of the antigen of both tests was estimated to have a molecular weight of 61,000 daltons by polyacrylamide gel electrophoresis.

Key words: diagnosis, EHV-1, ELISA, titration of antibody

Equine herpesvirus type 1 (EHV-1) causes acute respiratory disease in foals, abortion in mares, and occasionally neurologic disease [2, 7, 9–11, 15]. EHV-1 was introduced to Japan with infected mares from USA in 1967, prior to which only EHV-4 had been established in the horse population in Japan [6]. Subsequently, EHV-1 infection has become one of the most important causes of abortion in Japan [8]. In the Miho and Ritto Training Centers (TC) of the Japan Racing Association (JRA), respiratory disease caused by EHV-1 occurs almost every winter. By our epizootic research from 1980 to 1985, it was appeared that 25% of horses with fever were caused by viral infections, of which 75% were caused by EHV-1 infection [17, 18].

Agar gel immunodiffusion test (AGID) is currently used for diagnosis of EHV-1 infection in clinics of TC of JRA. It is possible to diagnose the disease using this test within 5 to 7 days after the horse manifests symptoms. Furthermore, it can be used in forecasting disease in individual horses, since a horse that demonstrates low precipitating antibody in the autumn has a risk of developing EHV-1 infection in the following season [19]. However, AGID is time consuming because it requires at least 18 hrs before results can be read. On the other hand, the enzyme-linked immunosorbent assay (ELISA) shows high sensitivity, reproducibility, low cost and suitability for rapid processing of large numbers of samples. It is currently widely used to detect antibodies to many viruses including EHV-1 [3–5], although there are few reports because field application of the test is assumed.

JRA is using ELISA that is manipulated by a robot system for screening of equine infectious anemia (EIA) during quarantine [16]. We have attempted to adapt this system for titration of antibody to EHV-1 in place of AGID.

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Materials and Methods

Serum samples: More than 50 samples that showed antibody level of −, +, ++ or ++++, respectively by AGID were randomly selected from about 2,000 samples that were collected in the Ritto TC in March, 1991.

Serum samples were taken from an experimentally inoculated horse with the HH-1 strain of EHV-1 through nasal route. The horse showed pyrexia and nasal discharge from 2 to 5 days after inoculation.

ELISA:

Antigen preparation: The HH-1 strain of EHV-1 was inoculated to Vero cells. Eagle’s MEM supplemented with 2% bovine serum albumin (Cohn V, BSA) was used as maintenance medium. Infected cells showing rounded-cell cytopathic effect (CPE) were harvested before cell lysis. Cells were collected by centrifugation at 100 × g for 5 min and cells were washed 5 times by 100 volumes of phosphate buffered saline (PBS). The pellet was then suspended in 10 volumes of 1% Triton X-100 and sodium deoxycholate in PBS and incubated in an ice bath for 10 min. Following centrifugation at 5,000 × g for 20 min the supernatant was used as antigen for ELISA. C-type ELISA plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with 50 µl of appropriate concentration of antigen diluted with 0.05 M carbonate buffer (pH 8.6) at 4°C for 18 hr. The plates were lyophilized and stored at 4°C till use.

ELISA: The ELISA method was used in the indirect method. Conditions used for ELISA were as mentioned in ELISA for EIA [16], as we planned to manipulate the ELISA system presented here by the same robot (Biomek 1000 and Sideloadier, Beckman Instruments, Inc., Fullerton, California, USA) for screening of the EIA in the TC. Briefly, the plate surfaces were blocked by 1% gelatin. The serum samples diluted to 1:808 by PBS supplemented with 1% BSA and 0.05% Tween 20 were added to wells in duplicate. After incubation at 37°C for 40 min, HRPO-conjugated anti horse IgG (H+L) (Bethyl Laboratories, Inc., Montgomery, Texas, USA) was added. After incubation for 20 min, TMBBlue (TCI-CDP, Milford, Massachusetts, USA) was added and incubated for a further 10 min. Color development was stopped by addition of 0.1 M H2SO4. Optical density of the samples was measured at 450 nm (OD450). Optimal antigen concentration was determined by the box titration by ELISA. An antibody negative, and a strongly positive serum to EHV-1 were taken from an experimentally infected horse before and 88 days after inoculation, respectively. A reference serum that is utilized in AGID was also used [16, 19]. Sera diluted to 1:808 as mentioned above were reacted to serially 2- or 3-fold diluted antigen. Antigen concentration was adjusted to that OD450 of the negative serum was 0.1 to 0.3, that of the strongly positive serum was 1.5 to 2.0, and that of the reference serum was 1.0.

AGID:

Antigen was made from Vero cells infected with the HH-1 strain. Virus and cell debris were precipitated from the culture medium by polyethylene glycol #6000 at 10% (w/w) and sodium chloride at 3% (w/w). Following centrifugation at 5,000 × g for 30 min the precipitate was suspended in 1% sodium deoxycholate and Nonidet P-40 in 0.1 M Tris-HCl buffered saline (pH 7.4). Subsequent procedures are as described for ELISA antigen.

The test was conducted as mentioned in previous reports [16, 19]. Briefly, the test was performed in a 1% agar plate in 0.15 M NaCl. The reaction was carried out in a moisture chamber at room temperature for 24 hr. Levels of precipitating antibody were judged by the location of precipitation line and expressed as − to +++.

Complement fixation (CF) test:

Antigen was concentrated from infected Vero cells with the same method for AGID. Following centrifugation at 5,000 × g for 30 min the precipitate was sonicated at 20 k Hz. for 30 sec. Comparative CF was conducted by the CH50 method as mentioned previously [18]. In this method, 50 µl of 5 units of guinea pig complement, 25 µl of 4 units of antigen, 25 µl of serum samples that were serially 2-fold diluted from 1:4, and 50 µl of 1.7% sensitized sheep red blood cells were used. Antibody titers were expressed as the reciprocal of the highest dilution that inhibited hemolysis by 50%.

Serum neutralization test (NT):

A mixture of serially 2-fold diluted serum and the HH-1 strain (100 TCID50/100 µl), was incubated in 96-well microtiter plates in duplicate for 60 min at 37°C, and subsequently supplemented with MDBK cells. Antibody titers were expressed as the reciprocal of the highest dilution that inhibited CPE.

Statistical analysis:

Correlation coefficients among OD450 of ELISA, antibody titers of AGID, CF and NT of each serum sample were calculated by Spearman’s method using StatView software (Abacus Concepts, Inc., Berkley, California, USA) for Macintosh computers.

Polyacrylamide gel electrophoresis (PAGE):

The molecular weight (Mw) of the antigens used for ELISA, AGID and CF were estimated by PAGE. The
sample was electrophoretically separated in 10% SDS-PAGE gels purchased by ACI Japan, Inc. (Yamato-shi, Kanagawa) at 15 mA constant current/cm. Molecular weight markers (RAINBOW PROTEIN MOLECULAR WEIGHT MARKERS, Amersham Japan, Tokyo) comprised rabbit myosin (200,000 daltons), phosphorylase b (92,500 daltons), BSA (69,000 daltons), ovalbumin (46,000 daltons), carbonic anhydrase (30,000 daltons), trypsin inhibitor (21,500 daltons) and lysozyme (14,300 daltons) were run in parallel. Following electrophoresis, gel was stained in Coomassie brilliant blue R250 (0.1% w/v) in 30% methanol: 10% acetic acid.

Results

Correlation between ELISA and other serological tests: A scatter graph of ELISA OD\textsubscript{450} and each precipitating antibody level of serum samples are shown in Fig. 1. Number of samples that showed precipitating antibody level of −, +, ++ and +++ was 53, 110, 83 and 78, respectively. The mean OD\textsubscript{450} value ± standard deviation of them was $0.27 \pm 0.12$, $0.43 \pm 0.22$, $1.10 \pm 0.34$ and $1.38 \pm 0.26$, respectively. The correlation coefficient among them was 0.842 and p value was <0.0001 as shown in Table 1.

A scatter graph of ELISA OD\textsubscript{450} and each CF antibody level of serum samples is shown in Fig. 2. Number of samples that showed CF antibody level of <1:4, 1:4, 1:8, 1:16, 1:32, 1:64 and ≥1:128 was 37, 67, 64, 49, 46, 47 and 14. The mean OD\textsubscript{450} value ± standard deviation of them was $0.28 \pm 0.17$, $0.37 \pm 0.15$, $0.51 \pm 0.31$, $1.02 \pm 0.33$, $1.28 \pm 0.29$, $1.37 \pm 0.27$ and $1.41 \pm 0.34$. The correlation coefficient among them was 0.826 and p value was <0.0001 as shown in Table 1.

A scatter graph of ELISA OD\textsubscript{450} and each NT antibody level of serum samples is shown in Fig. 3. Number of

<table>
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<th>Test</th>
<th>Correlation coefficient</th>
<th>p value</th>
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<td>AGID</td>
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<tr>
<td>CF</td>
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<td>NT</td>
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samples that showed NT antibody level of <1:2, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and ≥1:128 was 5, 14, 9, 8, 12, 16, 22 and 14. The mean OD450 value ± standard deviation of them was 0.34 ± 0.37, 0.11 ± 0.04, 0.21 ± 0.17, 0.38 ± 0.24, 0.52 ± 0.17, 0.53 ± 0.15, 0.58 ± 0.19 and 0.55 ± 0.20, respectively. The correlation coefficient among them was 0.603 and p value was <0.0001 as shown in Table 1.

Changes of antibody levels in an experimentally inoculated horse: As shown in Fig. 4, OD450, AGID and CF antibodies were 0.29, – and <1:4, respectively before inoculation. At 7 days post inoculation (DPI), they were 0.85, +++ and 1:4. The maximum antibody titers, 2.41, +++ and 1:32 were observed at 30 DPI.

Mw of antigens for ELISA, AGID and CF: Patterns of PAGE analysis are shown in Fig. 5. Electrophoresis of ELISA and AGID antigens revealed 10 bands of Mw from 38,000 to 200,000 daltons. The Mw of the major band was estimated to be 61,000 daltons. The CF antigen showed 26 bands of Mw from 14,000 to 210,000 daltons.

Discussion

It has been suggested that ELISA can be used in place of AGID for titration of antibody to EHV-1 and diagnosis of the disease, as OD450 measurements of ELISA and precipitating antibody levels in AGID are highly correlated. It is expected that the ELISA system will be practically used in clinics of TC for control of EHV-1 infection through titration of antibody to the virus. EHV-1 infection is the major infectious disease in TC affecting the racing schedule and training of racehorses almost every year [17, 18]. An epizootiological survey demonstrated that the majority of infected horses with EHV-1 were 3-year old which had shown low levels of precipitating antibody, – or + by AGID, in the preceding autumn [19]. It is possible to get results within 3 hr by ELISA although AGID needs more than 18 hr. AGID is not suitable for processing large number of samples. Infection with EHV-1 can be prevented by vaccination, isolation from infected horses and disinfection. Therefore, it is recommended that antibody levels of every new racehorse should be checked by ELISA, and horses showing low antibody level should be vaccinated.

ELISA OD450 levels and precipitating antibody levels were highly correlated. This was considered to be due to the similarity in the procedures for antigen preparation for ELISA and AGID. These antigens were dissolved by detergents from infected cell debris and concentrated viruses. By PAGE analysis, the antigens were constructed with almost one component which was suggested to be VP 19, one of the major components of the nucleocapsid, as the Mw was estimated as 61,000 daltons [1]. ELISA
using this protein is also expected to be used for titration of antibody to EHV-4, as antigenicity of the protein is common to EHV-1 and EHV-4 [19].

On the other hand, correlation between the OD 450 of ELISA and CF antibody level was smaller than that of between ELISA and AGID. A suggested reason for this was that the CF antigen includes intact virus particle and components, because 26 bands, Mw from 14,000 to 210,000 daltons, were observed by PAGE analysis. The correlation coefficient among OD450 of ELISA and NT antibody levels was very small. The reason for the low correlation was considered to be because the proteins reacting on NT were glycoproteins on the envelope of the virus [1, 12–14].

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References