Enzyme-Linked Immunosorbent Assay for Serological Survey of Equine Arteritis Virus in Racehorses

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ABSTRACT. To examine antibodies against equine arteritis virus (EAV), an enzyme-linked immunosorbent assay (ELISA) using purified virus antigen was developed. The results of ELISA were compared with those of serum neutralization (SN) tests. The ELISA absorbance values and the SN titers in sera collected weekly from EAV-infected horses showed a similar pattern. The ELISA could detect antibody to EAV in horses experimentally infected with not only a homologous virus strain, which was used as the ELISA antigen, but also a heterologous strain. Using the ELISA, serum samples collected in 1996 from racehorses in three prefectures (Hokkaido, Ibaraki, and Shiga) were examined and there was no evidence of recent EAV infection among these racehorse populations in Japan. The ELISA should be a simple and highly specific method for rapid screening of EAV infection in racehorses. — KEY WORDS: ELISA, equine arteritis virus, serological survey.

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Equine arteritis virus (EAV) which is a causative agent of equine viral arteritis (EVA), a member of the genus *Arterivirus*, causes not only respiratory disease in horses but abortion in pregnant mares [4]. EAV is widely distributed in horse populations throughout the world. In 1993, an outbreak of EVA occurred for the first time in the United Kingdom [12]. The source of infection was a horse imported from an Eastern European country. In the same year, racehorses suffered from EAV in the United States, and the origin of this incidence was also from a horse imported from Europe [7]. Although EAV infection has never been reported in Japan [6, 8, 11], some horses imported from abroad have possessed antibodies against EAV [1, 6]. These cases emphasize the importance of quarantine for imported horses.

The serum neutralization (SN) test is at present performed as the international standard test for the definitive diagnosis of EAV infection in many countries. The SN test is a reliable method but it takes several days to obtain results. Enzyme-linked immunosorbent assay (ELISA) is a simple and rapid method for screening a large number of samples and has been applied to the detection of antibody to EAV in horses using crude virus preparation [9] and recombinant viral protein [2] as antigens. In the present study, we developed an ELISA using highly purified virus antigen to detect antibodies against EAV and applied this method to a serological survey of sera recently collected from thoroughbred horses in Japan.

A modified Bucyrus strain of EAV [10], was used as the ELISA antigen. Culture supernatant of virus-infected RK-13 cells was added with polyethyleneglycol (PEG) 6,000 to a final concentration of 8% (w/v) and stirred overnight at 4°C. Two hours before centrifugation, NaCl was added to the fluid at a final concentration of 0.4 M. After centrifugation at 9,000 rpm for 30 min at 4°C, the resulting pellet was resuspended in TEN buffer (10 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 1 mM EDTA). The suspension was applied to a 30% sucrose cushion and centrifuged at 40,000 rpm for 2 hr. A virus band was collected and further purified by CsCl gradient and subsequent 20–50% sucrose gradient ultracentrifugation. The virus was collected by ultracentrifugation and treated with 0.5% Triton X-100 in phosphate-buffered saline at 4°C for 1 hr. The antigen was stored at −80°C until use. The antigen was diluted at a protein concentration of 1 μg/m*l* in 0.05 M carbonate-bicarbonate buffer (CB, pH 9.6), then 50 μ*l* of the antigen was coated on each well of a 96-well flat-bottom plate and left overnight at 4°C. After excess antigen was removed, the wells were blocked with 200 μ*l* of phosphate buffered saline (PBS) containing 5% fetal bovine serum (5F-PBS) at 37°C for 1 hr. After washing three times with PBS containing 0.02% Tween 20 (PBS-T), 50 μ*l* of each serum diluted at 1: 100 was added in duplicate to wells and incubated at 37°C for 1 hr. The plates were washed five times with PBS-T. Fifty μ*l* of horseradish peroxidase-conjugated anti-horse IgG antibody (Cappel, U.S.A.) was added to each well, and the plates were incubated at 37°C for 1 hr. The plates were washed and then 100 μ*l* of 0.05 M citrate buffer (pH 4.0) containing 0.2 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma, U.S.A.) and 0.004% hydrogen peroxide, was added to each well as substrate. After incubation at room temperature for 30 min, the absorbance was measured at 415 nm.

The serum neutralization (SN) test was carried out by the 50% plaque reduction method using Bucyrus strain according to the method of Fukunaga *et al.* [5]. Serum samples of thoroughbred horses, aged 2 to 6 years, were collected from 3 prefectures (Hokkaido, Ibaraki and Shiga) in Japan in 1989 and 1996, and sera obtained from imported riding horses in 1988 and 1989 [6] were also used. Sera collected weekly from two horses experimentally infected with Bucyrus and 84Ky-A1 strains of EAV, were also used.

In preliminary experiments, crude virus antigen, which was prepared from the supernatant of EAV-infected cells by polyethyleneglycol precipitation as described previously [9], showed weak reaction to SN positive sera (data not

shown) in ELISA. We used detergent-solubilized purified virus as the ELISA antigen in the present experiments.

To determine the positive-negative threshold absorbance for the experiments, a total of 257 serum samples which were negative for SN tests, collected in Ibaraki prefecture, were tested. The mean absorbance value of these sera was 0.087 with a standard deviation of 0.037. Therefore absorbance of 0.198 (0.087 + 3 × 0.037) or higher was taken as positive.

The results of the detection of antibody to EAV in horses using ELISA and SN test are compared in Table 1. Seven out of eight SN positive sera were considered as ELISA positive. This indicated that ELISA sensitivity to detect SN positive sera was 87.5%. The ELISA specificity in detecting SN negative samples as ELISA negative was 98.9% (376 out of 380). And the coincidence of the results of ELISA and SN test was 98.7%.

Serum samples collected from two experimentally infected horses were examined by ELISA and SN tests. As shown in Fig 1, the ELISA absorbance values increased after virus infection and were correlated with the SN antibody titers. Both horses seroconverted in the ELISA between 1 and 2 weeks postinfection. The result also indicated that the ELISA could detect antibodies of horses infected with a heterologous virus strain.

The sera collected from three distinct areas in 1996 were examined by ELISA. As shown in Table 2, 12 out of 839 samples (1.4%) showed positive reactions, but all these sera were negative in SN test.

Lang and Mitchell [9] first applied ELISA using crude virus antigen to detect antibody to EAV in Ontario racehorses. The sensitivity and specificity of ELISA to SN test were 100 and 91.4%, respectively. Chirnside *et al.* [2] showed the sensitivity and specificity of their ELISA, in which a recombinant glutathione-S-transferase fusion protein expressing amino acids 55–98 of EAV GL glycoprotein was used as antigen, were 99.6 and 90.1%, respectively. The coincidence of ELISA and SN test in these reports were 91.4 and 91.0%, respectively. The specificity of the ELISA described here (98.9%) was higher than those reported previously. On the other hand, the sensitivity of the ELISA described here (87.5%) was relatively low. This may be due to the small number of SN positive sera. Up till now, we have found only eight sera from imported riding horses were positive in the SN test. It is not clear whether these horses had been infected by, or vaccinated for EAV, or not. Cook *et al.* [3] reported that sera which showed false-positive reactions in the ELISA for EAV had reacted with bovine serum components introduced by vaccination. Although the majority of horses described here had been periodically vaccinated, the false-positive rate was very low and the specificity of the ELISA using the antigen of purified virus was very high (Table 1). The process of high purification of the virus could reduce the contamination of cellular or serum components to the antigen. In Western blotting, sera from horses which were experimentally infected with EAV, strongly reacted with viral nucleocapsid (N) protein as well as G_L glycoprotein (data not shown). Treatment of the purified virus with detergent could disrupt the virion and effectively reveal N protein to be recognized by sera in the ELISA. This may also contribute to the increase of the specificity of the assay described here.

Table 1. Comparison of antibody detection by ELISA and SN test

	Number of sera[a)]		Total
	SN positive	SN negative	
ELISA positive	7	4	11
ELISA negative	1	376	377
Total	8	380	388

a) SN positive sera were collected from imported riding horses and SN negative sera were collected from racehorses in 1989.

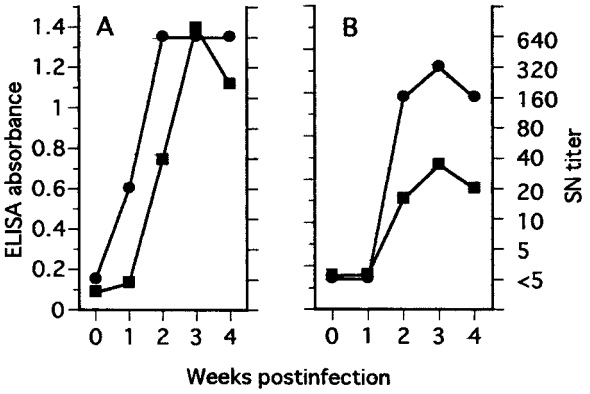

Fig. 1. Antibody responses of the horses experimentally infected with EAV. Horses were infected with (A) Bucyrus strain and (B) 84 Ky-A1 strain of EAV. ■: ELISA absorbance value (415 nm), ●: SN antibody titer.

Table 2. Serological survey of antibodies to EAV in sera of racehorses

Prefecture	Number of sera[a)]		Total
	ELISA negative	ELISA positive (SN positive)	
Hokkaido	331	4 (0)	335
Ibaraki	257	2 (0)	259
Shiga	239	6 (0)	245
Total	827	12 (0)	839

a) Sera were collected from racehorses in 3 prefectures in 1996.

A serological survey of EVA in Japan was first reported by McCollum and Bryans [11]. They investigated 304 sera collected in 1970 and 1971 and there was no evidence of EAV infection among horses in Japan. Later, Konishi *et al.* [8] detected no antibody against EAV from any serum samples collected from 107 horses in 1972 and 1973. Akashi *et al.* [1] detected twelve seropositive horses out of

140 horses imported in 1973 and 1974. Fukunaga *et al*. [6] reported that a serological survey of 1656 horse sera collected between 1988 and 1990 was conducted, and that only eight imported riding horses possessed antibodies to EAV. In the present study we confirmed these earlier studies. We could not show any evidence of recent EAV infections among racehorse populations in Japan.

The ELISA described here is highly specific and should be useful for quarantine and epidemiological surveys for EAV infection.

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