Diagnosis and Sero-Epizootiology of Equine Herpesvirus Type 1 and Type 4 Infections in Japan Using a Type-Specific ELISA

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(Received 26 January 1998/Accepted 5 June 1998)

ABSTRACT. Recently, a type-specific ELISA using equine herpesvirus type 1 (EHV-1) and type 4 (EHV-4) glycoprotein Gs (gGs) was developed by Crabb and Studdert [1993]. To investigate the dissemination of EHV-1 and -4 among horses in Japan, we applied their ELISA as suitable for discriminating between EHV-1 and -4 infections serologically. Type-specificity of the ELISA was confirmed by using paired sera of infected horses with either EHV-1 or -4. Application of the ELISA to sera collected before and after the winter season of 1995-1996 from 80 racehorses revealed that 30 horses showed significant antibody responses against EHV-1 and 9 against EHV-4, respectively. The results indicated that this ELISA using paired sera is useful for a diagnosis and an epizootiological study on EHV-1 and -4 infections. — **KEY WORDS:** diagnosis, epizootiology, equine herpesvirus.

J. Vet. Med. Sci. 60(10): 1133-1137, 1998

Equine herpesvirus types 1 (EHV-1) and 4 (EHV-4) are causative agents of equine rhinopneumonitis. Both viruses belong to the family *Herpesviridae*, the subfamily *Alphaherpesvirinae*. The viruses have been shown a wide geographic distribution and cause serious economic losses worldwide. EHV-1 causes acute upper respiratory disease, central nervous system disorders and contagious virus abortion in horses, and EHV-4 causes respiratory disease and, occasionally, abortion [1]. The difference between EHV-1 and -4 has been demonstrated in pathogenicity in baby mice [11-13], restriction endonuclease analysis of viral DNA [18], and polymerase chain reactions [2, 6, 15]. However, because of the extensive antigenic cross-reactivity, it is difficult to distinguish between EHV-1 and -4 infections by conventional serological tests.

In Japan, only EHV-4 in horses before 1967 was distributed [4, 16]. An occurrence of EHV-1 abortion among imported mares in 1967 caused the first outbreak of EHV-1 abortion among pregnant mares raised in Japan [5]. Thereafter both EHV-1 and -4 spread among various horse populations in Japan. Today, many racehorses at two Training Centers of the Japan Racing Association (JRA) have suffered from respiratory disease with pyrexia by EHV-1 or -4 infection [20]. Furthermore, foals and yearlings suffering from respiratory disease on breeding and rearing farms also have been diagnosed serologically to be infected with EHV-1 or -4 [8, 19]. The virological study revealed that EHV-1 caused epizootics of respiratory disease among racehorses exclusively in the winter season, and that EHV-4 also caused respiratory disease among racehorses sporadically and among foals and rearing horses irrespective of the season [10].

Virological diagnosis can identify the type of infected virus, but they are time-consuming and laborious. Therefore, it is considered to be very important to diagnose the type of infected virus serologically. The purpose of this study was to develop a type-specific serological test for diagnosis of EHV-1 and -4 infections, which facilitates the elucidation of epizootiological characteristics of both infections among the various horse populations in Japan.

The EHV-1 89c25p strain [9] and an EHV-4 strain TH20, the Japanese prototype of EHV-4 [4], were used in this study. Viruses were propagated at low multiplicities of infection in primary fetal horse kidney cells. Infected cells showing a cytopathic effect were treated with 1% sodium dodecyl sulfate (SDS) and proteinase K (0.1 mg/ml) in 0.1 M Tris-HCl (pH 8.0), 0.1M NaCl, 5 mM EDTA at 37°C overnight. Following the DNA extraction with phenol and chloroform-isoamyl alcohol (24:1), ethanol precipitated preparations were dissolved in water.

Crabb and Studdert [3] reported that type-specific, continuous epitopes were located in the corresponding C-terminal variable region of EHV-1 gG (amino acids 288 to 350) and EHV-4 gG (amino acids 287 to 382), which show little amino acid identity. To express the type-specific antigen, the bacterial expression plasmid pGEX-5X1 was used. The recombinant pGEX-5X1 plasmids, pGEX-gG1P and pGEX-gG4P, were constructed according to the methods described previously [3] with a minor modification. (i) pGEX-gG1P was constructed by PCR amplification (35 cycles of 92°C for 1 min, 55°C for 1 min, 72°C for 1 min) with the EHV-1 89c25p DNA as the template and the following primers: forward primer, gG1P-F, containing an *EcoRI* site, 5'-CCGAATTCGGTGACGAAACATACGA-3', and reverse primer, gG1P-R, containing an *XhoI* site, 5'-AACTCGAGCTGGATGCCGTTTCGACG-3'. The amplified fragment was digested with *EcoRI* and *XhoI* and cloned into the pGEX-5X1 digested with the same restriction endonucleases. (ii) pGEX-gG4P was constructed by PCR amplification (35 cycles of 92°C for 1 min, 50°C for 1 min,

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72°C for 1 min) with the EHV-4 TH20 DNA as the template and following primers: forward primer, gG4P-F, containing an *Eco*RI site, 5'-CCGAATTCGAAAATGAAACCTACAG-3', and reverse primer, gG4P-R, containing an *Xho*I site, 5'-AACTCGAGGAGGCCATGCAACACAC-3'. The amplified fragment was cloned into the pGEX-5X1 in the same manner with pGEX-gG1P. *Escherichia coli* XL1-blue strains containing the correct recombinant plasmids were identified, and glutathione S-transferase (GST) fusion proteins were purified by a method based on that of Smith and Johnson [17].

For analysis of the expression products, SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis were carried out. SDS-PAGE was carried out with the discontinuous Laemmli buffer system [7]. All samples were dissolved in the buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, and 0.001% bromophenol blue), and then disrupted by heating for 2 min at 100°C. Polypeptides were separated on a 15% SDS-PAGE. The gel was stained with 0.006% Coomassie Brilliant Blue in 10% acetic acid. The result showed that the major fusion proteins produced by pGEX-gG1P and pGEX-gG4P had molecular weights (MWs) of approximately 37 and 36 kDa respectively (data not shown). These sizes are similar to the predicted sizes of GST-gG1P and GST-gG4P fusion proteins. Next, in order to examine the type-specificity of the expressed fusion proteins, immunoblot analysis using sera collected from experimentally infected horses with EHV-1 or -4 was carried out. The fusion proteins, GST-gG1P and GST-gG4P, specifically reacted with EHV-1-infected foal serum and EHV-4-infected yearling serum, respectively (data not shown). It was confirmed that the variable region of EHV-1 and -4 gGs used in this study contained type-specific epitopes, as reported previously [3].

Crabb and Studdert have reported a single-dilution type-specific ELISA using these recombinant gG fusion proteins [3]. To improve their ELISA method for serological diagnosis of EHV-1 and -4 infections to simplify the estimation of test results, we used serially diluted paired sera. The procedure for the ELISA is as follows: GST fusion or GST protein diluted in the adsorption buffer (0.05 M Carbonate-bicarbonate buffer pH 9.6) were dispensed into the wells of flat-bottomed 96-well microplates (Flow Laboratories, Inc., U.S.A.) in 50 µl aliquotes. The plates were covered and stored at 4°C overnight for adsorption of antigen. The plates were replaced with 100 µl of PBS containing 0.1% bovine serum albumin for blocking. After incubation at 37°C for 60 min, the blocking solution was discarded and 50 µl of the test serum, serially diluted in PBS containing 0.05% Tween 20 (PBST) supplemented with 10% calf serum (CS), was added to each well. After incubation at 37°C for 60 min, the plates were washed 6 times with PBST, and then 50 µl of horse radish peroxidase (HRP) labeled goat anti-horse IgG antibody (Chemicon International, Inc., U.S.A.), diluted to 1:1,000 in PBST supplemented with 10% CS, was added to each well. After incubation for 60 min at 37°C, the plates were washed 6

times with PBST, and then a substrate solution, containing 0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H₂O₂ and 0.3 mg/ml of 2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate], was added to each well in 100 µl aliquotes. The absorbance at 405-nm was read after incubation for 30 min, using a micro-ELISA reader. The ELISA titer was expressed as the reciprocal of the maximum dilution that showed an ELISA value, which is the absorbance difference between the GST fusion protein and GST protein, equal to or over 0.1.

To determine the most appropriate concentration of the GST fusion proteins to use as ELISA antigens for antibody detection, box titration was carried out using EHV-1 or -4 infected horse serum. A concentration of 0.2 µg/ml was indicated as the most appropriate for both types of GST fusion protein (data not shown). Hereafter, GST and GST fusion proteins were used at the concentration of 0.2 µg/ml.

To examine the availability of this ELISA system, sera collected from horses which were experimentally infected with EHV-1 or -4 were applied to this system (Fig. 1). Serum samples were collected from three foals (No. 1–3; correspond to R-1 - R-3 in reference 9) which were experimentally inoculated with EHV-1 89c25p gE-gI-rev [9], similar pathogenicity and virus growth to the parent strain, 89c25p, and three yearlings (No. 4–6) which were experimentally inoculated with EHV-4 TH20 strain. Figure 1A exhibited that 14 days after EHV-1 infection, sera of horses showed over 4-fold higher titers against EHV-1 gG than preinfection sera, but less than 2-fold higher titers against EHV-4. In spite of no detectable level of virus neutralizing (VN) antibody, the ELISA could detect the rise of antibody against EHV-1 (Fig. 1, No. 2). Figure 1B exhibited that 14 days after EHV-4 infection, sera of horses showed over 16-fold higher titers against EHV-4 gG than preinfection sera, but less than 2-fold higher titers against EHV-1. These results suggested that this ELISA method is type-specific and possesses a high sensitivity. From these results, we tentatively defined that EHV-1 or -4 infection is diagnosed by 4-fold rise of ELISA titer between paired sera.

For further analysis of the specificity, we examined the ELISA titers of paired sera collected at acute and convalescent phases from 16 febrile racehorses (No. 1–16) in which a tentative serological diagnosis of EHV-1 or -4 infection was made by complement fixation (CF) test. The result showed that a final diagnosis of EHV-1 infection was made in 8 horses and that diagnosis of EHV-1 infection might be made in another 3 horses (Table 1). On the other hand, no racehorse was diagnosed as EHV-4 infection. We also carried out ELISA on paired sera collected from 9 foals and rearing horses (No. 17–25) with pyrexia from which EHV-4 strains were isolated. In 7 horses, ELISA titers against EHV-4 gG rose significantly, but they did not in 2 foals, No. 17 and No. 18 (Table 1). Since these two foals were under three-months of age, the maternal antibody from colostrum might have affected their antibody responses to the EHV-4 infection. These results suggested that this

Fig. 1. ELISA titers of sera collected from experimentally infected horses with EHV-1 or EHV-4. (A) ELISA titers of sera collected from experimentally infected foals with EHV-1. (B) ELISA titers of sera collected from experimentally infected yearlings with EHV-4. Closed and open columns show VN titers against EHV-1 and EHV-4, respectively. Closed and open circles show ELISA titers against EHV-1 and EHV-4 gGs, respectively.

ELISA method might be useful for a definitive diagnosis of EHV-1 and -4 infections.

For epizootiological study on EHV-1 and -4 infections among racehorses in Japan, sera were collected before and after the winter season of 1995–1996 from 80 racehorses at Ritto and Miho Training Centers of the JRA (Table 2). ELISA titers against EHV-1 gG significantly rose in 30 (37.5%) out of 80 horses and those against EHV-4 gG rose in 9 (11.25%). These results were consistent with previous reports that EHV-1 is responsible for epizootic respiratory diseases in racehorses in winter [10, 20], and revealed that EHV-4 is also sporadically responsible for respiratory disease among racehorses during the period. In addition, sera of two horses (Table 2, M28 and M42) reacted to both antigens. Since most of the EHV-1 or -4 infected horses judged by the ELISA (35 out of 37, 94.6%) showed an antibody rise only against one of the antigens in their sera, it was suggested that the two horses were infected with both EHV-1 and -4 at a certain time or times during the period between the time points of sample collection.

In this study, it became possible to make a definitive diagnosis of EHV-1 or -4 infection using paired sera.

However, in some cases, results of ELISA did not consist with those of CF test. We speculated that because of different antigens between gG fusion proteins for ELISA and whole extracts of EHV-1-infected cells for CF test, reactivity of antibody might be different, or that antigenicity of certain field viruses might be different from that of 89c25p used in this study. Furthermore, ELISA titers against EHV-4 gG were higher than those against EHV-1 gG in most sera including the sera from experimentally infected horses, although similar VN antibody titers against the homologous virus were observed in those sera (see Fig. 1). Therefore, it is thought that immunogenicity of the EHV-4 gG might be higher than that of the EHV-1 gG. Further analysis would be required to resolve these problems.

In conclusion, the ELISA method using paired sera and judging by a 4-fold rise of antibody titer between paired sera might be useful both for diagnosis and for epizootiological studies on EHV-1 and -4 infections, which had been previously thought to be difficult to carry out serologically.

Table 1. ELISA titers of paired sera collected from horses with pyrexia

Horse ^{a)}	Date of collection		ELISA titers against			
			EHV-1		EHV-4	
	no.	acute	convalescent	acute	convalescent	acute
1	Mar/17/1996	Apr/19/1996	20	80**	160	160
2	Mar/23/1996	Apr/12/1996	<20	320**	1280	2560
3	Mar/7/1996	Mar/27/1996	<20	20*	320	320
4	Feb/28/1996	Mar/15/1996	<20	40**	160	160
5	Apr/8/1996	Apr/26/1996	<20	20*	1280	1280
6	Jan/19/1996	Feb/9/1996	40	80	640	320
7	Jan/30/1996	Feb/16/1996	40	320**	640	320
8	Feb/8/1996	Feb/23/1996	<20	80**	160	320
9	Feb/9/1996	Mar/1/1996	<20	160**	320	320
10	Mar/24/1996	Apr/12/1996	40	40	>2560	2560
11 ^{b)}	Jan/4/1989	Jan/15/1989	<20	20*	320	160
12	Jan/5/1989	Jan/21/1989	20	40	320	640
13	Jan/6/1989	Jan/21/1989	160	320	1280	640
14 ^{c)}	Jan/6/1989	Jan/22/1989	20	640**	160	160
15	Jan/9/1989	Jan/25/1989	640	640	1280	2560
16 ^{c)}	Jan/11/1989	Jan/23/1989	40	160**	1280	1280
17 ^{d)}	Jan/3/1987	Jul/8/1987	40	40	40	40
18 ^{d)}	Jun/3/1987	Jul/8/1987	20	20	160	80
19 ^{d)}	Jul/8/1987	Sep/1/1987	20	20	320	2560**
20 ^{d)}	Jul/7/1987	Sep/1/1987	<20	<20	160	1280**
21 ^{d)}	Jul/8/1987	Sep/1/1987	20	20	<20	320**
22 ^{d)}	Feb/10/1988	Mar/9/1988	<20	20	160	1280**
23 ^{d)}	Feb/10/1988	Mar/9/1988	40	40	640	>2560**
24 ^{d)}	Oct/11/1988	Nov/11/1988	<20	20	160	>2560**
25 ^{d)}	Oct/12/1988	Oct/26/1988	20	40	320	>2560**

a) No. 1–16; racehorses, No. 17–25; foals and rearingmares. b) EHV-1 was isolated from the horse. c) Nervous disorders were observed in the horse. d) EHV-4 was isolated from the horse. **, Significant antibody response. *, Probably significant antibody response.

ACKNOWLEDGEMENT. We thank the veterinary staffs of the JRA for collecting serum samples.

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Table 2. ELISA titers of sera collected from racehorses before and after the winter season of 1995–1996

ELISA titers against					ELISA titers against						
Horse	Sex	EHV-1		EHV-4		Horse	Sex	EHV-1		EHV-4	
no.		Oct, 1995	May, 1996	Oct, 1995	May, 1996	no.		Oct, 1995	May, 1996	Oct, 1995	May, 1996
R1		20	<20	2560	1280	M1		80	1280**	2560	640
R2		40	20	2560	2560	M2		<20	160**	40	40
R3		40	40	2560	2560	M3		80	40	320	1280**
R4		40	40	640	>2560**	M5		40	80	640	320
R5		20	<20	1280	1280	M6		<20	80**	640	640
R6		80	80	2560	2560	M7		160	160	320	1280**
R7		20	320**	1280	1280	M8		20	320**	80	40
R8		80	1280**	>2560	2560	M9		<20	<20	640	320
R9		20	80**	1280	1280	M11		40	320**	320	320
R10		20	<20	2560	2560	M12		20	20	320	640
R11		40	320**	1280	1280	M13		80	160	640	1280
R12		20	320**	1280	640	M14		80	160	640	1280
R13		160	80	2560	>2560 (**)	M15		80	160	320	320
R14		<20	<20	320	160	M16		20	20	640	640
R15		40	40	>2560	>2560 (-)	M17		20	80**	640	320
R16		160	320	2560	>2560 (**)	M18		20	<20	2560	640
R17		160	80	>2560	>2560 (-)	M19		20	80**	160	320
R18		20	320**	2560	2560	M20		1280	640	320	640
R19		20	<20	>2560	2560	M21		320	160	640	640
R20		20	20	>2560	>2560 (-)	M22		<20	320**	640	320
R21		80	320**	2560	2560	M23		40	40	640	640
R22		320	>2560**	>2560	2560	M24		40	80	320	40
R23		80	320**	2560	1280	M25		160	640**	640	640
R24		40	40	640	640	M26		160	160	640	320
R25		40	160**	640	640	M27		20	640**	160	320
R26		<20	20	2560	>2560 (-)	M28		80	320**	640	2560**
R27		20	320**	1280	1280	M29		320	640	160	160
R28		80	40	320	2560**	M30		640	640	640	1280
R29		40	320**	2560	2560	M31		40	20	640	640
R30		40	40	2560	2560	M32		40	1280**	2560	2560
R31		40	160**	>2560	2560	M33		160	160	1280	640
R32		40	20	>2560	1280	M35		160	80	640	640
R33		80	80	1280	>2560**	M36		80	160	320	320
R34		20	40	1280	2560	M37		80	160	640	640
R35		40	20	2560	1280	M38		160	160	320	640
R36		40	40	>2560	2560	M40		40	160**	2560	>2560 (-)
R37		320	320	>2560	2560	M41		40	320**	1280	640
R38		40	20	>2560	>2560 (-)	M42		80	>2560**	320	1280**
R39		40	40	1280	1280	M43		160	640**	640	1280
R40		20	160**	80	160	M44		40	80	320	320

** Significant antibody response. (**) Significant antibody response indicated by ELISA using more highly diluted sera. (-) No significant antibody response indicated by ELISA using more highly diluted sera. R; Racehorses in Ritto Training Center of the Japan Racing Association. M; Racehorses in Miho Training Center of the Japan Racing Association.