NOTE *Virology*

Improvement of an enzyme-linked immunosorbent assay for equine herpesvirus type 4 by using a synthetic-peptide 24-mer repeat sequence of glycoprotein G as an antigen

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ABSTRACT. To increase the sensitivity of an enzyme-linked immunosorbent assay (ELISA) for equine herpesvirus type 4 (EHV-4) that uses a 12-mer peptide of glycoprotein G (gG4-12-mer: MKNNPIYSEGSL) [4], we used a longer peptide consisting of a 24-mer repeat sequence (gG4-24-mer: MKNNPIYSEGSLMLNVQHDDSIHT) as an antigen. Sera of horses experimentally infected with EHV-4 reacted much more strongly to the gG4-24-mer peptide than to the gG4-12-mer peptide. We used peptide ELISAs to test paired sera from horses naturally infected with EHV-4 (n=40). gG4-24-mer ELISA detected 37 positive samples (92.5%), whereas gG4-12-mer ELISA detected only 28 (70.0%). gG4-24-mer ELISA was much more sensitive than gG4-12-mer ELISA.
KEY WORDS: EHV-4, glycoprotein G, peptide ELISA

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Equine herpesvirus type 1 (EHV-1) and type 4 (EHV-4) are major causes of respiratory disease in horses [1]. Pyrexias caused by EHV-1 infection are commonly observed in winter in the training centers of the Japan Racing Association (JRA), especially in 2- to 3-year-old horses [6]. In contrast, respiratory diseases caused by EHV-4 infection are observed mainly in young horses on breeding and rearing farms [6]. Although these viruses have antigenic cross-reactivity, ELISAs that use the C-terminal type–specific region of glycoprotein G (gG) as an antigen and can distinguish infections with the two viruses [2] have been developed. To detect EHV-1- and EHV-4-infected horses, we currently use ELISAs that utilize recombinant gG proteins modified for Japanese isolates (rgG1 ELISA and rgG4 ELISA) [7].

The type-specific region of gG of the EHV-4 TH20p strain, which has previously been chosen as representative of Japanese isolates, consists of 93 amino acids containing two identical repeat sequences [4, 7]. Detailed analysis of the repeat sequence consisting of 24 amino acids (MKNNPIYSEGSLMLNVQHDDSIHT) has revealed that an epitope is located in the 12 amino acids MKNNPIYSEGSL (gG4-12-mer) [4]; this same study showed the potential of an ELISA using the synthetic peptide of gG4-12-mer as an antigen [4]. Although another, 11-mer, epitope consisting of MKNNPVYSESL (gG4-11-mer) has been found in the repeat sequences of other EHV-4 isolates, the synthetic peptide of this sequence has less reactivity to EHV-4-infected sera than does the gG4-12-mer peptide [5]. These studies suggested that gG4-12-mer ELISA could be a useful alternative to rgG4 ELISA. (The amino acid sequences of these synthetic peptides are summarized in Table 1.) However, when we applied gG4-12-mer ELISA to a large number of field samples, it seemed to be less sensitive than the currently used rgG4 ELISA in detecting EHV-4-infected horses. Here, we investigated the potential use of a longer peptide consisting of a 24-mer repeat sequence of gG (gG4-24-mer) as an antigen in peptide ELISA for diagnosing EHV-4-infection.

First, to compare the reactivities of EHV-4-infected sera to the synthetic peptides, we performed peptide ELISAs using gG4-12-mer [4, 5] and gG4-24-mer as antigens to test a series of sera from foals experimentally infected with EHV-4 (n=6, 28 to 63 days old at the time of virus inoculation). The foals were deprived of colostrum for 24 hr after birth; they had no history of infection with EHV-1 or EHV-4 or of inoculation with any kind of vaccine before viral inoculation. They were intranasally inoculated with EHV-4 strain TH20p (1×10^6 plaque-forming units/head). Sera were collected on days 0, 7, 14, 21 and 28. All experimental procedures were approved by the Animal Care Committee of the Equine Research Institute of the Japan Racing Association. Peptide ELISAs were performed as described previously, with minor modifications [4, 5]. Fifty microliters of synthetic peptide (Life Technologies Japan, Tokyo, Japan) diluted with 0.05 M carbonate-bicarbonate buffer (pH 9.6) at a concentration of 10 μg/m*l* was seeded onto a 96-well plate (Nunc Maxisorp, Thermo Scientific, Roskilde, Denmark). As blank controls, wells without peptide were also prepared. The plates were incubated at 37°C for 2 hr and then stored at 4°C overnight. After removal of the antigen solution, the wells were treated with 100 μl of a diluent consisting of phosphate-buffered saline (PBS) containing 10% bovine serum (Gibco, Grand Is-

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Table 1. Amino acid sequences of synthetic peptides used in ELISAs in this study and in previous studies

Name	Amino acid sequence
gG4-12-mer	MKNNPIYSEGSL
gG4-24-mer	MKNNPIYSEGSLMLNVQHDDSIHT
gG4-11-mer	MKNNPVYSESL

land, NY, U.S.A.) for blocking. In each subsequent step, the plates were incubated at 37°C for 30 min and were washed three times between the steps with PBS containing 0.05% Tween20 (Wako Pure Chemicals Industries, Osaka, Japan). After reaction of the wells with sera serially diluted from 1:10 to 1:1,280 with the above-described diluent (50 μl/well), horseradish peroxidase–conjugated goat anti-equine IgG polyclonal antibody (MP Biomedicals, Inc., Aurora, OH, U.S.A.) diluted at 1:5,000 (50 μl/well) was added as a secondary antibody. Color development was performed with TMB peroxidase substrate (Moss, Inc., Pasadena, MD, U.S.A.), and the optical density (OD) at a wavelength of 450 nm was measured. Final OD values were derived by subtracting the values for wells without antigen from those for wells with antigen. The antibody titer was expressed as the reciprocal of the maximum serum dilution that gave OD values of 0.1 or more. The rgG4 ELISA was also performed as described previously, with minor modifications [7]. Serum dilution was started from 1:2,000 in rgG4 ELISA.

To compare the reactivity of the sera to each synthetic peptide, we plotted the OD values obtained at a serum dilution of 1:10 (Fig. 1). OD values higher than 2.0 are indicated as 2.0 on the graphs. The sera reacted to gG4-24-mer peptide much more strongly than to gG4-12-mer peptide in all horses, except foal #5. In foal #5, higher reactivity to gG4-24-mer peptide was apparent when the serum was diluted to 1:20 or more (data not shown). The antibody titers in rgG4 ELISA, gG4-24-mer ELISA and gG4-12-mer ELISA are indicated in Table 2. Overall, the titers in gG4-24-mer ELISA were much higher than those in gG4-12-mer ELISA. The day at which a significant titer rise (more than four-fold) was detected in rgG4 ELISA and in gG4-24-mer ELISA was identical in five out of six foals, while that in gG4-12-mer ELISA was slightly delayed (foal #2 and #3) or not detected throughout the experiment (foal #1, Table 2). In foal #3, gG4-24-mer ELISA detected significant titer rise at 21 days, while rgG4 ELISA failed to detect the titer rise. Therefore, detection of antibody responses in gG4-24-mer ELISA was almost equal to that in rgG4 ELISA, or more sensitive in some cases. Although the foals had been deprived of colostrum for 24 hr after birth, antibodies were detected on day 0 in some of the foals in rgG4 ELISA and in gG4-24-mer ELISA (Table 2). One possible explanation for the pre-existing antibodies might be the residual maternal antibodies transferred from the dams after lifting of colostrum deprivation. However, we cannot exclude the other possibility that the pre-existing antibodies might be due to non-specific reaction.

Next, we tested paired sera from horses naturally infected with EHV-4 (n=40) by using ELISAs with gG4-12-mer or gG4-24-mer peptides; the results of the ELISAs were then compared. Sera were collected from pyretic horses and from horses kept with them at either of the JRA's training centers or at JRA Hidaka Training and Research Center, the JRA Equine Research Institute, or other farms in Japan in the period from 2003 to 2015. Pre-sera were taken on the day on which the affected horses developed pyrexia, and post-sera were taken 14 to 28 days later. All post-sera had been confirmed by rgG4 ELISA to show a significant titer rise (more than four-fold) compared with the pre-sera. As negative controls, we tested paired sera taken from clinically healthy horses (n=10) about 1 month apart. Peptide ELISAs were performed as described above, except that serial dilution of the sera started at 1:20. Paired sera were judged positive for EHV-4-infection when a significant titer rise (more than four-fold) was detected. The statistical significance of the sensitivities of the ELISAs was analyzed by using a chi-squared test. A level of $P<0.05$ (Bonferroni corrected)

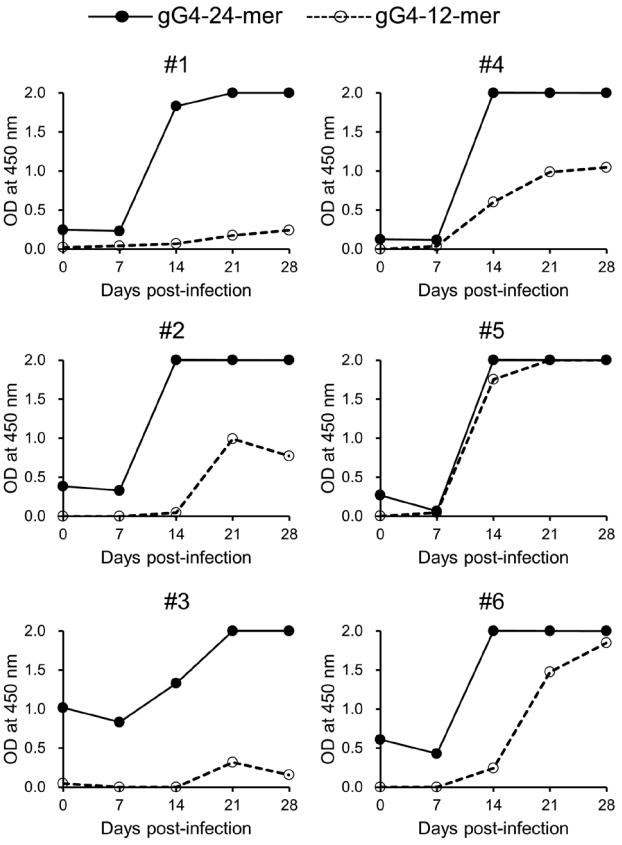

Fig. 1. Reactivity of sera from foals experimentally infected with EHV-4 to gG4-24-mer and gG4-12-mer peptides. Six foals were intranasally inoculated with EHV-4 strain TH20p (1×10^6 plaque-forming units/head). Sera collected on days 0, 7, 14, 21 and 28 were diluted at 1:10 and applied to wells coated with either gG4-24-mer or gG4-12-mer and to wells without peptides. Final OD values at a wavelength of 450 nm were obtained by subtracting values for wells without antigens from those for wells with antigens. OD values higher than 2.0 are indicated as 2.0.

Table 2. rgG4 ELISA, gG4-24-mer ELISA and gG4-12-mer ELISA titers of sera from foals experimentally infected with EHV-4

Foal	ELISA	Days post infection				
		0	7	14	21	28
1	rgG4	2,000	2,000	128,000	128,000	≥256,000
	gG4-24-mer	10	20	160	640	≥1,280
	gG4-12-mer	<10	<10	<10	10	10
2	rgG4	2,000	<2,000	8,000	32,000	32,000
	gG4-24-mer	20	20	640	≥1,280	≥1,280
	gG4-12-mer	<10	<10	<10	40	40
3	rgG4	8,000	4,000	4,000	8,000	8,000
	gG4-24-mer	80	40	160	640	640
	gG4-12-mer	<10	<10	<10	10	20
4	rgG4	<2,000	<2,000	16,000	32,000	32,000
	gG4-24-mer	10	10	≥1,280	≥1,280	≥1,280
	gG4-12-mer	<10	<10	40	80	80
5	rgG4	<2,000	<2,000	16,000	32,000	32,000
	gG4-24-mer	10	<10	≥1,280	≥1,280	≥1,280
	gG4-12-mer	<10	<10	160	320	160
6	rgG4	2,000	2,000	16,000	128,000	128,000
	gG4-24-mer	20	20	≥1,280	≥1,280	≥1,280
	gG4-12-mer	<10	<10	20	160	160

was considered significant. Cohen's kappa coefficient values were evaluated according to the guidelines of Landis and Koch [3], as follows: <0, no agreement; 0 to 0.20, slight; 0.21 to 0.40, fair; 0.41 to 0.60, moderate; 0.61 to 0.80, substantial; and 0.81 to 1.0, almost perfect agreement.

The results of each peptide ELISA and of rgG4 ELISA are given in Tables 3 and 4. gG4-12-mer ELISA detected only 28 out of 40 positive samples, with a sensitivity of 70.0% (Table 3). The results of gG4-12-mer ELISA agreed moderately well with those of rgG4 ELISA (κ=0.4828). In contrast, the rate of detection of EHV-4-infected horses was dramatically improved when we used gG4-24-mer peptide. gG4-24-mer ELISA detected 37 out of 40 positive samples, with a sensitivity of 92.5% (Table 4). This was significantly higher than that of gG4-12-mer ELISA (P<0.05). The results of gG4-24-mer ELISA agreed almost perfectly with those of rgG4 ELISA (κ=0.8315).

In our previous study, to identify the epitope region, we tested five synthetic peptides (each consisting of 12-mer peptides overlapping by eight to 10 amino acids) that covered the 24-amino-acid repeat sequence of EHV-4 gG [4]. EHV-4-positive sera reacted only to the gG4-12-mer peptide; almost no reaction was detected to the other peptides, which included residues on the C-terminal side (MLNVQHDDSIHT) [4]. However, the reactivity of the sera to the gG4-12-mer peptide was a little less than that to the recombinant protein of the 24-amino-acid sequence [4]. Our current results also support the possibility that one stretch of the repeat sequence contains an epitope or epitopes other than the one located in the gG4-12-mer peptide. Another possibility is that the residues on the C-terminal side contribute to the adsorption of gG4-24-mer peptide to the wells so that the epitope on the N-terminal side was set free and that might facilitate the strong reaction of the antibodies.

Table 3. Detection of natural EHV-4 infection in horses by using rgG4 ELISA and gG4-12-mer ELISA

		gG4-12-mer-ELISA		Total
		+	–	
rgG4-ELISA	+	28	12	40
	–	0	10	10
	Total	28	22	50

Table 4. Detection of natural EHV-4 infection in horses by using rgG4 ELISA and gG4-24-mer ELISA

		gG4-24-mer-ELISA		Total
		+	–	
rgG4-ELISA	+	37	3	40
	–	0	10	10
	Total	37	13	50

In conclusion, gG4-24-mer ELISA was more sensitive than gG4-12-mer ELISA; moreover, the agreement between the results of gG4-24-mer ELISA and the currently used rgG4 ELISA was almost perfect. Because it uses an easily prepared synthetic peptide, this method can be employed by diagnostic laboratories.

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