



Research paper

Development of an enzyme-linked immunosorbent assay for Getah virus infection in horses using recombinant E2 protein as an antigen



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ABSTRACT

Getah virus causes fever, skin eruptions, and limb edema in horses. For a high-throughput and time-saving method for serodiagnosis, we explored immunogenic antigens of Getah virus, and established an enzyme-linked immunosorbent assay (ELISA) using a recombinant protein. Western blot analysis using sera from infected horses showed strong reaction with viral antigens around 46 kDa corresponding to E1 or E2 glycoproteins. Recombinant E2 (rE2) protein reacted more strongly with infected horse sera than did rE1 protein in both Western blotting and ELISA. In ELISA using rE2 protein (rE2-ELISA), for all horses experimentally infected with Getah virus ($n = 7$), optical density (OD) exceeded the cutoff value at 14 days post-infection. ODs in five of nine vaccinated horses also slightly exceeded the cutoff value after vaccination. Among naturally infected horses ($n = 28$), 24 were seronegative in the acute sera, which turned seropositive in the convalescent sera. For the four horses seropositive in the acute sera, an endpoint method with serial dilutions of paired sera detected a ≥ 4 -fold increase in titer. In conclusion, we established rE2-ELISA that could detect horse antibodies against Getah virus after experimental and natural infections; this should be useful in the diagnosis and surveillance of Getah virus infection.

1. Introduction

Getah virus is classified in the genus *Alphavirus* in the family *Togaviridae*. It is mosquito-borne and is widespread from Eurasia to Australasia (Fukunaga et al., 2000). This virus causes fever, skin eruptions, and limb edema in horses (Fukunaga et al., 2000), and it causes fetal death and reproductive disorders in pigs (Izumida et al., 1988; Yago et al., 1987). The first outbreak of Getah virus infection in horses in Japan occurred in 1978 (Kamada et al., 1980); since then, Japan has experienced several outbreaks. The latest outbreaks occurred from 2014 to 2016 at one facility, affecting 33 horses in 2014, 30 horses in 2015, and 6 horses in 2016 (Nemoto et al., 2015; Bannai et al., 2016b; Nemoto et al., 2017).

Serological diagnosis of Getah virus infection in horses is based on virus-neutralization (VN), hemagglutinin inhibition (HI), and complement-fixation tests (Imagawa et al., 1992). The VN test requires live viruses and a cell culture system, and it takes 3 days to yield a result.

The other tests are also undesirable due to the laborious procedures involved in antigen preparation and pre-treatment of serum samples. As an alternative to such classical tests, enzyme-linked immunosorbent assays (ELISAs) using an extract of Getah virus-infected cells or purified virions have been used in serosurveillance of Getah virus among wild boars and pigs in Japan (Hohdatsu et al., 1990; Kuwata et al., 2018). Although those ELISAs showed correlation with VN or HI titers, the antigen prepared from the virus-infected cells tend to be of unstable quality, and the cutoff value needs to be optimized for each lot made. Therefore, a recombinant protein-based ELISA system would be preferable, as it may increase the accuracy of the test. In this study, we explored immunogenic antigens of the Getah virus, and established an ELISA system using recombinant proteins for the diagnosis of Getah virus infection in horses.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; rE1, recombinant E1; rE2, recombinant E2; OD, optical density; VN, virus-neutralization; HI, hemagglutinin inhibition; MEM, minimum essential medium; IPTG, isopropyl β -D-1-thiogalactopyranoside; RRV, Ross River virus; EEEV, eastern equine encephalitis virus; WEEV, western equine encephalitis virus; VEEV, Venezuelan equine encephalitis virus; PBS, phosphate-buffered saline; PBST, PBS containing 0.05% Tween 20; HRP, horseradish peroxidase; SD, standard deviation

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2. Materials and methods

2.1. Cell culture

For virus proliferation, Vero cells (Sumitomo Dainippon Pharma, Tokyo, Japan) and LLC-PK1 cells (American Type Culture Collection, Manassas, VA, USA) were used. For virus-neutralizing test, Vero cells were used. Vero cells were cultured in minimum essential medium (MEM; MP Biomedicals, Irvine, CA, USA) containing 10% fetal bovine serum (Sigma Aldrich Inc., St. Louis, MO, USA), 100 units/mL penicillin, and 100 µg/mL streptomycin (Sigma Aldrich Inc.). LLC-PK1 cells were cultured in medium 199 (Sigma Aldrich Inc.) containing 10% fetal bovine serum (Sigma Aldrich Inc.), 100 units/mL penicillin, and 100 µg/mL streptomycin (Sigma Aldrich Inc.). MEM or medium 199 containing 2% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin was used as a maintenance medium for virus proliferation and the VN tests.

2.2. Viruses

For antigen preparation for Western blotting and VN tests, Getah virus strain 14-I-605 was used. For the experimental infection, strains MI-110, 14-I-605, and 15-I-752 were used. These strains were isolated from affected horses during the outbreaks of Getah virus infection in the Japanese horse populations in 1978 (MI-110; Kamada et al., 1980), 2014 (14-I-605, Bannai et al., 2015), and 2015 (15-I-752, Bannai et al., 2016b).

2.3. Preparation of protein extract from Getah virus-infected cells

Getah virus strain 14-I-605 passaged three times in Vero cells was inoculated onto a 1-day monolayer of Vero cells or LLC-PK1 cells in a 75 cm² flask with a multiplicity of infection of 0.01. After 1 h incubation at 37 °C, the inoculum was replaced with the maintenance medium. When cytopathic effects appeared in more than 80% of the cells, the culture supernatant was removed and the cells were treated with 1 mL of RIPA buffer (Thermo Scientific, Rockford, IL, USA) containing Protease Inhibitors Cocktail (Nacalai Tesque, Kyoto, Japan) for 5 min at 4 °C. The lysed cells were harvested and were centrifuged at 14,000 × g for 15 min at 4 °C, and the supernatant was ultrafiltrated using an Amicon Ultra 10K Centrifugal Filter Unit (Merck-Millipore, Carrigtwohill, Ireland) to yield a protein concentration of 10 mg/mL. Mock-treated lysates of Vero cells or LLC-PK1 cells were also prepared as described above as a control.

2.4. Construction of expression plasmids

For expression of Getah virus proteins in *Escherichia coli*, nucleotide fragments corresponding to the E1 and E2 genes were amplified by RT-PCR from genomic RNA of Getah virus strain 14-I-605. Viral RNA was extracted from the supernatant of virus-infected Vero cells with a Qiagen Viral RNA Mini Kit (Qiagen Inc., Venlo, Netherlands). The primer sequences were as follows: E1-forward, 5'-ATTATTGAATTCTTACGAACACACCGCAACGATCC-3'; E1-reverse, 5'-ATTATTCTCGAGTCA GCGGCGCATAGTCCACAC-3'; E2-forward, 5'-ATTATTGAATTCTAGTGTGACGGAACACTTCAATG-3'; and E2-reverse, 5'-ATTATTCTCGAGTAGGCATGCGCTCGTGGTGCGC-3'. The forward primers contained an *EcoRI* site, and the reverse primers contained an *XhoI* site. The amplified fragments were purified with a BEX DNA Purification Kit I (BEX Co. Ltd, Tokyo, Japan), digested with *EcoRI* and *XhoI*, and then cloned into a bacterial expression plasmid (pET-47b; Merck KGaA, Darmstadt, Germany) digested with the same set of restriction enzymes. The DNA sequences were confirmed to be the same as the ones on the GenBank/EMBL/DBJ databases (accession number, LC079088).

2.5. Expression of recombinant proteins

The expression plasmids were transformed into *E. coli* BL21 (DE3) (Merck KGaA), and the transformed clones were cultured in LB medium containing 50 µg/mL kanamycin (Wako Pure Chemicals Industries, Osaka, Japan). Protein expression was induced by adding isopropyl β-d-1-thiogalactopyranoside (IPTG) (Sigma Aldrich Inc.) at 0.5 mM final concentration, and the culture was harvested after incubation for 4 h at 37 °C. Protein extraction and purification was performed by using a Capturem His-Tagged Purification Maxiprep Kit (Takara Bio Inc., Kusatsu, Japan) under denaturing conditions in the presence of 6M-guanidine hydrochloride (Wako Pure Chemicals Industries), followed by re-folding by dialysis. The protein concentration was measured using a BCA Protein Assay Kit (Thermo Scientific).

2.6. Horse sera

Sera collected from horses experimentally infected with Getah virus were used as positive controls in the Western blotting and for optimization of the ELISA protocol. Experimental inoculation of horses (1 year old, $n = 2$ for each strain) with strains MI-110 (horses #1 and #2) and 14-I-605 (horses #3 and #4) was performed in our previous study (Nemoto et al., 2016) by intramuscular injection of a 50% tissue culture infective dose of 1.3×10^5 in a volume of 2 mL. In the current study, three additional horses were inoculated with strain 15-I-752 in the same manner. All horses were healthy and had no serological evidence of previous Getah virus infection or vaccination (based on results of VN tests that are mentioned below; antibody titers were < 1:4 for antibodies to strain 14-I-605). Sera collected on Days 0 and 14 after viral inoculation were used. Sera from horses #1–#4 at Day 14 were used individually for the Western blots in Section 3.1. Pooled sera from horses #1–#4 at Days 0 and 14 were used for the Western blots in Section 3.1 and 3.2. Sera from horse #2 at Days 0 and 14 were used for the ELISA in Section 3.2 and 3.3. Serum from horse #3 at Day 14 was used as a standard in the ELISAs in Section 3.4 and after.

Negative control sera were collected from a horse population (1-year-old, $n = 30$) kept in Hokkaido Prefecture in northern Japan, an area free from Getah virus. The horses were healthy and had no serological evidence of previous Getah virus infection or vaccination. The sera were used in Section 3.4 for setting a cutoff value for the ELISA.

Vaccination of horses with Getah virus vaccine was performed according to the manufacturer's recommendation. Nine horses (1 year old) were intramuscularly inoculated with inactivated Getah virus vaccine (Nisseiken, Tokyo, Japan) two times with a 28-day interval. All horses were healthy and had no serological evidence of previous Getah virus infection or vaccination. Sera collected at the time of first vaccination (Day 0, V1), second vaccination (Day 28, V2), and at 28 days after the second vaccination (Day 56, V2 + 28) were used in the ELISA in Section 3.5.

Paired sera from horses naturally infected with Getah virus ($n = 28$) were collected during the outbreaks of Getah virus infection among racehorses from 2014 to 2016 in Japan. The horses were 2–7 years old (mean, 2.8 years old) and developed fever from summer to autumn in each year; they were confirmed to be infected with Getah virus by RT-PCR or VN testing, or both (Nemoto et al., 2015; Bannai et al., 2015, 2016b; Nemoto et al., 2017). The sera in the acute phase (acute sera) were collected when the horses developed fever, and sera in the convalescent phase (convalescent sera) were collected after a 2- to 10-week interval (mean, 31.8 days). Although they each had a history of Getah virus vaccination at least once before the disease onset, the influence of vaccination on the antibody response between paired sera was considered to be negligible, because a sufficient time period had passed from the latest vaccination to the time of disease onset (33–141 days, [mean, 99 days]).

2.7. Mouse immune ascitic fluids for various Alphaviruses

The ascitic fluids from mice immunized with Ross River virus (RRV), eastern equine encephalitis virus (EEEV), western equine encephalitis virus (WEEV) and Venezuelan equine encephalitis virus (VEEV) were purchased from American Type Culture Collection.

2.8. Western blotting

The lysate of Getah virus-infected cells (5 µg/lane) and recombinant proteins rE1 and rE2 (0.5 µg/lane) were electrophoresed on NuPAGE 10% Bis-Tris Gel (Life Technologies, Carlsbad, CA, USA) for 1 h at 100 V. The separated proteins were transferred to 0.2 µm polyvinylidene difluoride membranes (Trans-Blot Turbo Transfer Pack; Bio-Rad Laboratories, Hercules, CA, USA) using Trans-Blot Turbo (Bio-Rad Laboratories) for 7 min at 25 V. The membranes were treated with phosphate-buffered saline (PBS) with 5% skim milk overnight at 4 °C for blocking. A diluent consisting of PBS containing 0.05% Tween 20 (Wako Pure Chemicals Industries) (PBST) and 0.5% skim milk was used for the dilution of horse sera and secondary antibodies. After washing three times with PBS, the membranes were treated with horse sera diluted at 1:100 with the diluent for 1 h at room temperature. After washing three times with PBST, the membranes were treated with horseradish peroxidase (HRP)-conjugated goat anti-horse IgG (H + L) (Sigma Aldrich Inc.) diluted at 1:5000 with the diluent for 1 h at room temperature. After washing three times, the membranes were treated with ECL Western Blotting Substrate (Thermo Scientific) for 1 min at room temperature, and images were visualized by ChemiDoc XRS + with Image Lab Software (Bio-Rad Laboratories).

The numbers of amino acids were calculated for each viral protein from the genomic sequence information of strain 14-I-605 (GenBank accession number, [LC079088.1](https://www.ncbi.nlm.nih.gov/nuccore/LC079088.1); Nemoto et al., 2016), and the predicted molecular weight was calculated using the SIB ExpASY Bioinformatics Resources Portal (<https://www.expasy.org>; Artimo et al., 2012).

2.9. VN test

The VN test for Getah virus was performed using strain 14-I-605 as described previously (Bannai et al., 2015). Serial 2-fold diluted sera starting from a dilution of 1:4 were tested, and the VN titer was defined as the highest dilution that completely inhibited virus growth. Horses that showed a ≥ 4 -fold increase between the paired sera were defined as having seroconverted.

2.10. ELISA

A 96-well plate (Nunc Maxisorp; Thermo Scientific) was coated with the recombinant proteins diluted with 0.05 M carbonate-bicarbonate buffer (pH 9.6) at a protein concentration of 1.5 µg/mL. As blank controls, wells without antigen were also prepared. The plates were incubated at 37 °C for 2 h for antigen adsorption. After removal of antigen solution, the wells were treated with 100 µL of a diluent consisting of PBS containing 10% bovine serum (Gibco, Grand Island, NY, USA) for blocking. At each subsequent step, the plates were incubated at 37 °C for 30 min and washed three times with PBST. After reaction of the wells with sera serially diluted from 1:20 to 1:320 with the above-described diluent (50 µL/well), HRP-conjugated goat anti-horse IgG (H + L) (Sigma Aldrich Inc.) diluted at 1:5000 (50 µL/well) was added as a secondary antibody. Color development was performed with TMB peroxidase substrate (Moss, Inc., Pasadena, MD, USA), followed by addition of a stopping solution (0.25 N H₂SO₄, Wako Pure Chemicals Industries). The optical density (OD) at 450 nm was measured. Final OD values were derived by subtracting the values for wells without antigen from those for wells with antigen.

For the assays using immune ascitic fluids for RRV, EEEV, WEEV and VEEV, series of dilutions from 1:20 to 1:640 were tested by the

ELISA as described above. Paired sera from Getah virus-infected horse (horse #2) were used as positive control, and the purified mouse IgG1 antibody (Bethyl Lab., Inc., Montgomery, TX, USA) was used as a negative control. After the antigen-coated wells were treated with the ascitic fluids or horse sera, they were treated with HRP-conjugated protein A/G (Thermo Scientific) diluted at 1:2000 as a secondary antibody, followed by the color development described above.

A tentative cutoff value was calculated from the OD values of negative control sera ($n = 30$) as the mean + 3 standard deviations (SDs). For standardization of the cutoff point in each assay, serial dilutions of standard serum (Day 14 of horse #3) starting from 1:40 to 1:1280 were tested with the negative controls, and the optimal dilution corresponding to the cutoff value was calculated from the regression curve.

In the single dilution method, horse sera were diluted to 1:80, and if the serum showed OD values over the cutoff value, it was regarded as positive. A negative result with the acute serum and a positive result with the convalescent serum was regarded as indicating seroconversion. In the endpoint method, serial dilutions of horse sera from 1:80 to 1:10,240 were tested, and the antibody titer was expressed as the maximum serum dilution that gave OD values of 0.1 or more. A ≥ 4 -fold increase between the paired sera was regarded as indicating seroconversion.

3. Results

3.1. Immunogenicity of viral proteins to sera from horses infected with Getah virus

To assess the immunogenicity of viral proteins, lysate of Getah virus-infected Vero cells was tested by Western blotting using sera from horses infected with Getah virus ($n = 4$). The lysate of virus-infected Vero cells showed signals around 30 kDa, 46 kDa, and 57 kDa that were absent or faint in the control cell lysate (Fig. 1A). From the molecular weights of viral proteins predicted from the genome sequence (Table 1), it seemed the 30 kDa signal was a capsid protein, the 46 kDa signal was glycoproteins E1 or E2, and the 57 kDa signal was non-structural protein 1 (nsP1) or 3 (nsP3). The 46 kDa signal was the strongest among them, and was observed in all four blots treated with individual sera (Fig. 1A). However, weak signals corresponding to this molecular weight were also observed in the lysate of mock-infected Vero cells, especially when the blot was treated with the serum from horse #1. For further confirmation of immunogenicity of 46 kDa antigen, virus-infected or mock-infected lysates were prepared using another cell line, LLC-PK1. The 46 kDa band was obvious in the lysate of virus-infected LLC-PK1 cells, while no signal was detected in the corresponding molecular weight in the control cell lysate (Fig. 1B). Thus, the 46 kDa band in virus-infected cells represents the reaction with Getah virus E1 or E2 glycoproteins, rather than the reaction with host proteins. Because of the high reactivity in the Western blot, E1 and E2 proteins were selected as promising antigens for the ELISA.

3.2. Reactivities of recombinant E1 and E2 proteins with respect to sera from Getah virus-infected horses

The nucleotide sequences corresponding to the Getah virus E1 and E2 proteins were amplified by RT-PCR, and were cloned into the pET-47b vector. The recombinant E1 (rE1) and E2 (rE2) proteins expressed by *E. coli* had molecular weights corresponding to those predicted from amino acid sequences, namely 47.5 kDa for rE1 and 46.3 kDa for rE2 (Suppl. Fig. 1). The recombinant proteins were tested by Western blotting to see their reactivities with respect to sera from virus-infected horses. Both proteins showed bands in the predicted molecular weights described above, although smeared signals also appeared in a range covering the lower molecular weights (Fig. 1C). Compared to the rE1 protein, the molecular weight of the rE2 protein was closer to the one that appeared on the lysate of virus-infected cells and the signal was

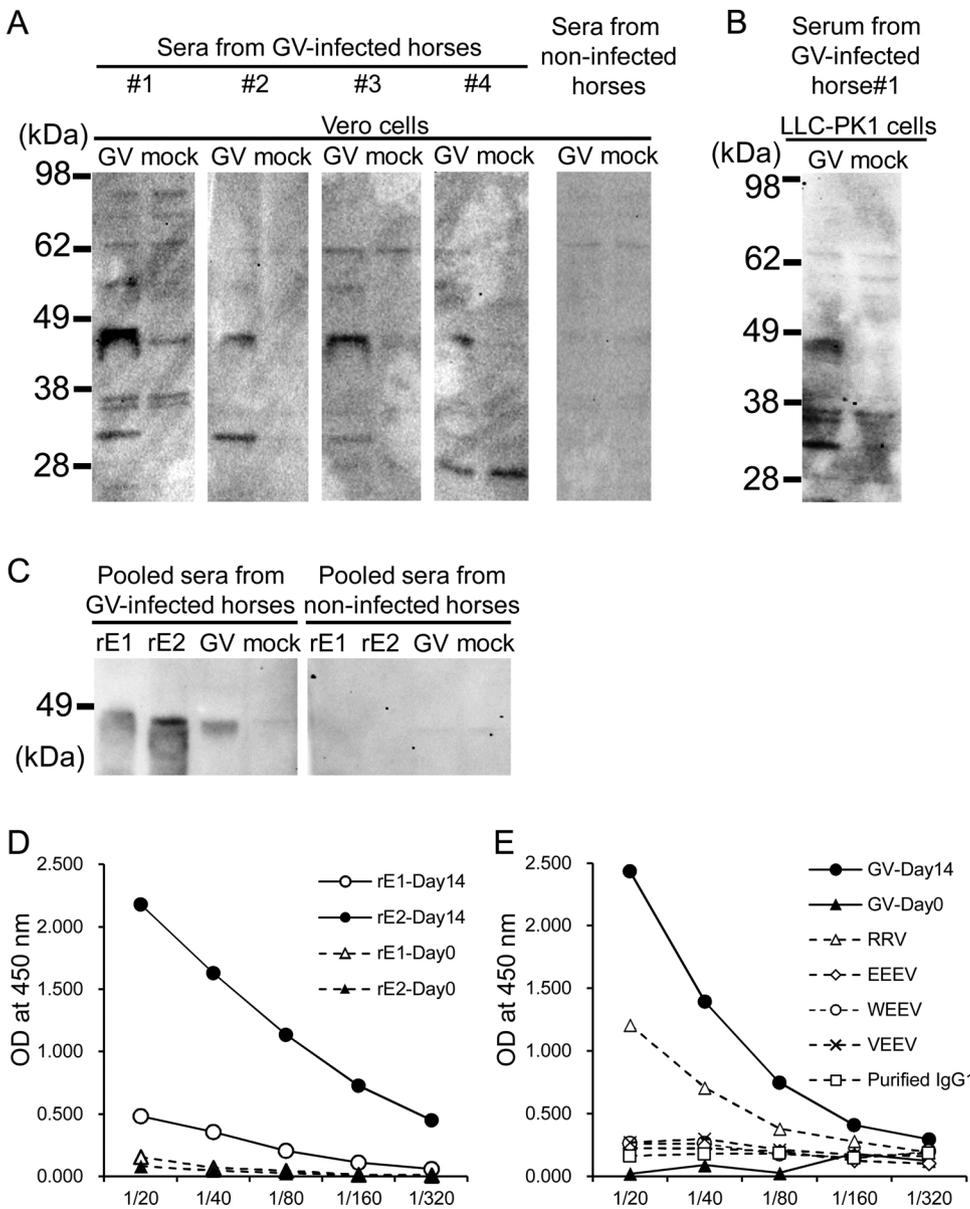


Fig. 1. Reactivity of serum from Getah virus-infected horses with respect to viral antigens. (A). Western blots showing reactivity of sera from experimentally infected horses with respect to viral antigens. Lysates of Getah virus-infected Vero cells (GV) and mock-infected cells (mock, 5 µg/lane) were electrophoresed on 10% polyacrylamide gel, and were transferred to 0.2 µm polyvinylidene difluoride membranes. Membranes were treated with horse sera from experimentally infected horses (Day 14 of horses #1–#4, individually) or non-infected horses (Day 0 of horses #1–#4, pooled) followed by HRP-conjugated goat anti-horse IgG (H + L). The reaction was visualized with ECL Western Blotting Substrate (Thermo Scientific). (B) Western blot showing reactivity of a serum from an experimentally infected horse (Day 14 of horse #1) with respect to viral antigens prepared from Getah virus-infected LLC-PK1 cells. Lysates of Getah virus-infected LLC-PK1 cells (GV) and mock-infected cells (mock, 5 µg/lane) were electrophoresed and tested by Western blotting as described above. (C) Western blots showing reactivity of sera from experimentally infected horses with respect to recombinant E1 (rE1) and E2 (rE2) proteins. The proteins (0.5 µg/lane) were electrophoresed and tested by Western blotting as described above. Lysates of Getah virus-infected cells (GV) and mock-infected cells (mock, 5 µg/lane) were used as controls. Sera from Getah virus-infected horses (Day 14 of horses #1–#4, pooled) or from non-infected horses (Day 0 of horses #1–#4, pooled) were used. (D) Reactivity of rE1 and rE2 proteins to sera from an experimentally infected horse (horse #2) by ELISA. 96-well plates were coated with the proteins (5 µg/mL). After adsorption and blocking, the wells were treated with the Getah virus-infected horse serum (Day 14 of horse #2) or non-infected serum (Day 0 of horse #2) serially diluted from 1:20 to 1:320, followed by HRP-conjugated goat anti-horse IgG (H + L) polyclonal antibody. Color development was performed with TMB peroxidase substrate and the reaction was stopped using 0.25 N H₂SO₄. The OD at 450 nm was

measured. Final OD values were derived by subtracting the values for wells without antigen from those for wells with antigen. (E) Reactivity of rE2 protein to antibodies against RRV, EEEV, WEEV, VEEV by ELISA. A 96-well plate was coated with the rE2 protein (5 µg/mL). After adsorption and blocking, the wells were treated with the Getah virus-infected horse serum (Day 14 of horse #2), non-infected serum (Day 0 of horse #2), mouse ascitic fluids against RRV, EEEV, WEEV or VEEV, and purified mouse IgG1 serially diluted from 1:20 to 1:320, followed by HRP-conjugated protein A/G. Color development, measurement and calculation of final OD values was performed as described above.

Table 1
Predicted amino acid lengths and molecular weights of viral proteins of Getah virus strain 14-I-605.

Viral proteins	Amino acids	Molecular weight (kDa)
Non-structural proteins		
nsP1	534	59.3
nsP2	798	89.7
nsP3	522	57.7
nsP4	611	68.6
Structural proteins		
capsid	268	30.1
E3	64	7.2
E2	422	46.3
6K	61	6.7
E1	438	47.5

much stronger (Fig. 1C). Thus, the 46 kDa signal that appeared on the lysate of virus-infected cells was considered to indicate the reaction between native E2 glycoprotein and the antibodies against it.

The reactivities of these recombinant proteins with respect to serum from a virus-infected horse were also confirmed by ELISA. The rE2 protein showed high reactivity with respect to serum from the virus-infected horse in a serum dilution series with excellent contrast with the serum from a non-infected horse, while rE1 only showed weak reactivity in a few points of higher concentration in serum from an infected horse (Fig. 1D). From this result, rE2 was selected as the ELISA antigen, and the established ELISA is designated as rE2-ELISA in the following sections.

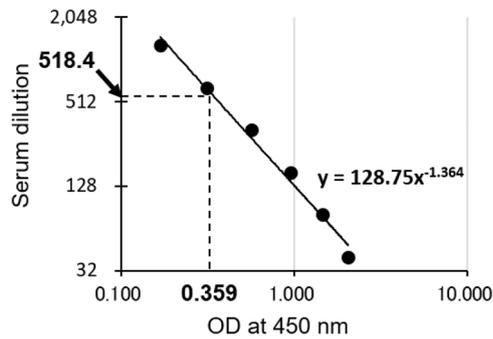


Fig. 2. Calculation of standard serum dilution equivalent to the tentative cutoff value in the rE2-ELISA. Serial dilutions of standard serum (Day 14 of horse #3) starting from 1:40 to 1:1280 were tested by the rE2-ELISA. The tentative cutoff value calculated from the negative control sera (0.359, $n = 30$) was equivalent to that given by the standard serum diluted to 1:518.4.

3.3. Reactivities of recombinant E2 protein with respect to ascitic fluids from mice immunized with RRV, EEEV, WEEV and VEEV

To assess the possible cross-reactivities of the rE2 protein with antibodies against various *Alphavirus* species, ascitic fluids from mice immunized with RRV, EEEV, WEEV and VEEV were tested in the rE2-ELISA. The reaction between the rE2 protein and a serum from Getah virus-infected horse was detected by HRP-conjugated protein A/G (Fig. 1E), with a similar range of OD values as the reaction using HRP-conjugated anti-horse IgG (H + L) antibodies (Fig. 1D). The rE2 protein reacted with ascitic fluid against RRV in a dose-dependent manner, while such reaction was not observed with those against other *Alpha-virus* species (Fig. 1E).

3.4. Calculation of cutoff value for rE2-ELISA

To set the cutoff value for the rE2-ELISA, negative control sera ($n = 30$) collected from a horse population free from Getah virus were tested. All had been confirmed as antibody-negative in the VN test. The OD values in the rE2-ELISA ranged from 0.000 to 0.316 with a mean \pm SD of 0.125 ± 0.078 . As a result, the tentative cutoff value (mean + 3 SDs) was calculated to be 0.359. In this experiment, a serial 2-fold dilution of a standard serum (Day 14 of horse #3) was tested to draw a standard curve for conversion of the cutoff value into a standard serum dilution (Fig. 2). The formula of the curve ($y = 128.75x^{-1.364}$) showed the OD value of 0.359 was equivalent to that given by the standard serum diluted to 1:518.4. In the experiments described below, to minimize the negative influence caused by variation between test batches, the same standard set was tested in each time, and the OD value of the standard serum diluted to 1:518.4 was defined as the cutoff value.

3.5. Detection of antibodies by rE2-ELISA in sera from horses experimentally infected with Getah virus and horses vaccinated with Getah virus vaccine

Paired sera from horses ($n = 7$) experimentally infected with Getah virus were tested. None of the horses had detectable VN antibodies at Day 0, and 1:32–1:128 titers were detected at Day 14 (Table 2). In the rE2-ELISA, the cutoff value applied to this batch was 0.352. All but horse #2 had OD values below the cutoff value at Day 0, and all horses showed obvious seroconversion at Day 14 (Fig. 3A and Table 2), with the OD values ranging from 0.853 to 1.708. Horse #2 also showed a more than 2-fold increase in OD values (0.798 to 1.708) between the paired sera (Fig. 3A and Table 2). The horses inoculated with MI-110 and 14-I-605 strains at Day 14 showed OD values ranging from 1.271 to 1.708, whereas those inoculated with 15-I-752 strain showed relatively

Table 2

VN titers and ELISA results for serum samples collected from horses experimentally infected with Getah virus.

Horse #	Virus strain	VN titers		ELISA OD values	
		Day 0	Day 14	Day 0	Day 14
1	MI-110	< 1:4	1:128	0.104	1.397*
2	MI-110	< 1:4	1:128	0.798*	1.708*
3	14-I-605	< 1:4	1:128	0.079	1.701*
4	14-I-605	< 1:4	1:128	0.066	1.271*
5	15-I-752	< 1:4	1:32	0.128	1.064*
6	15-I-752	< 1:4	1:64	0.084	1.004*
7	15-I-752	< 1:4	1:64	0.064	0.853*

* OD at 450 nm higher than the cutoff value (0.352).

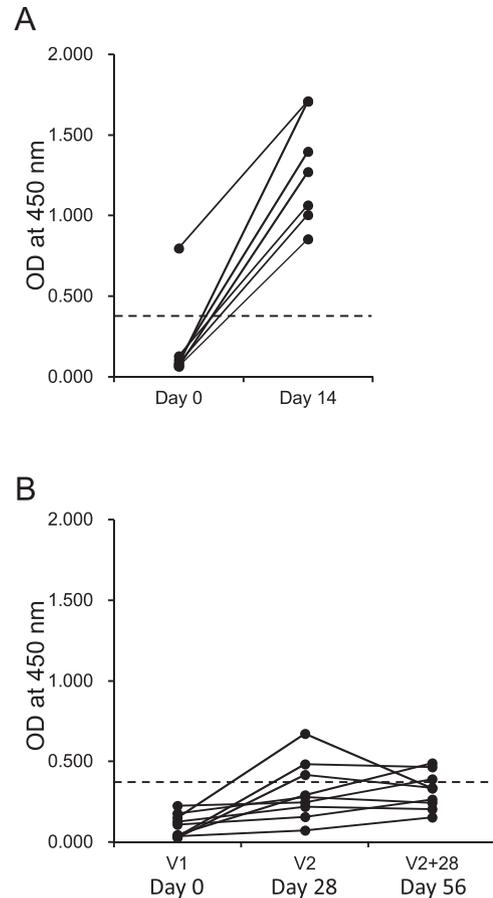


Fig. 3. rE2-ELISA for sera from horses experimentally infected with Getah virus and those vaccinated with Getah virus vaccine. (A) Paired sera from experimentally infected horses ($n = 7$). (B) Sequential sera from vaccinated horses ($n = 9$). Dashed line, cutoff value (0.352).

low OD values (0.853 to 1.064) (Table 2).

Sera from horses ($n = 9$) vaccinated with Getah virus vaccine were also tested. No horse had detectable VN antibodies at Day 0 (V1) or Day 28 (V2), and five horses showed 1:4–1:16 titers at Day 56 (V2 + 28) (Table 3). In the rE2-ELISA, all of them had OD values below the cutoff value at Day 0 (Fig. 3B and Table 3). After the first vaccination, three horses (#9, #11, and #14) seroconverted at Day 28, and two additional horses (#8 and #15) did so at Day 56 (Fig. 3B and Table 3). In these five horses, OD values higher than the cutoff value ranged from 0.389 to 0.671, with a mean \pm SD of 0.486 ± 0.090 .

Table 3

VN titers and ELISA results for the serum samples collected from horses vaccinated with Getah virus vaccine.

Horse #	VN titers			ELISA OD values		
	Day 0 (V1)	Day 28 (V2)	Day 56 (V2 + 28)	Day 0 (V1)	Day 28 (V2)	Day 56 (V2 + 28)
8	< 1:4	< 1:4	1:16	0.047	0.292	0.489*
9	< 1:4	< 1:4	1:16	0.028	0.419*	0.337
10	< 1:4	< 1:4	< 1:4	0.127	0.219	0.205
11	< 1:4	< 1:4	1:16	0.035	0.485*	0.466*
12	< 1:4	< 1:4	< 1:4	0.035	0.073	0.154
13	< 1:4	< 1:4	1:8	0.177	0.280	0.244
14	< 1:4	< 1:4	1:16	0.149	0.671*	0.334
15	< 1:4	< 1:4	< 1:4	0.226	0.246	0.389*
16	< 1:4	< 1:4	< 1:4	0.109	0.156	0.266

V1, first vaccination; V2, second vaccination.

* OD at 450 nm higher than the cut-off value (0.352).

Table 4

VN titers and ELISA results for serum samples collected from horses naturally infected with Getah virus.

Horse #	VN titers		ELISA OD values		ELISA titers	
	Acute	Convalescent	Acute	Convalescent	Acute	Convalescent
17	< 1:4	1:64	0.096	1.873*	ND	ND
18	< 1:4	1:256	0.034	2.099*	ND	ND
19	< 1:4	≥ 1:512	0.216	2.775*	ND	ND
20	< 1:4	1:256	0.048	2.030*	ND	ND
21	< 1:4	1:128	0.234	1.426*	ND	ND
22	< 1:4	≥ 1:512	0.106	1.271*	ND	ND
23	< 1:4	1:32	0.037	1.203*	ND	ND
24	< 1:4	1:16	0.059	0.964*	ND	ND
25	1:4	1:128	0.331	1.480*	ND	ND
26	< 1:4	1:256	0.071	1.233*	ND	ND
27	< 1:4	1:64	0.033	1.871*	ND	ND
28	< 1:4	≥ 1:512	0.162	2.021*	ND	ND
29	1:4	1:32	0.041	0.534*	ND	ND
30	< 1:4	1:8	0.062	2.002*	ND	ND
31	< 1:4	1:64	0.145	1.227*	ND	ND
32	< 1:4	1:16	0.141	0.964*	ND	ND
33	< 1:4	1:128	0.300	1.872*	ND	ND
34	< 1:4	1:256	0.296	1.415*	ND	ND
35	< 1:4	1:64	0.210	1.568*	ND	ND
36	< 1:4	1:64	0.243	1.836*	ND	ND
37	< 1:4	1:256	0.096	1.563*	ND	ND
38	< 1:4	≥ 1:512	0.080	2.733*	ND	ND
39	< 1:4	≥ 1:512	0.120	1.989*	ND	ND
40	< 1:4	1:16	0.030	1.115*	ND	ND
41	< 1:4	1:256	1.739*	3.000*	1:1280	1:5120
42	< 1:4	1:128	0.415*	1.353*	1:160	1:640
43	1:4	≥ 1:512	0.715*	3.000*	1:320	1:5120
44	< 1:4	1:256	0.451*	2.641*	1:160	1:2560

ND, not done.

* OD at 450 nm higher than the cut-off value (0.352).

3.6. Detection of antibodies by rE2-ELISA in sera from horses naturally infected with Getah virus

Paired sera from horses ($n = 28$) naturally infected with Getah virus were tested. All horses had VN titers equal to or lower than 1:4 in the acute sera, and 1:8 to $\geq 1:512$ titers in the convalescent sera (Table 4). In the rE2-ELISA, the cutoff value applied to this batch was 0.352. Out of 28 horses, 24 had OD values below the cutoff value in the acute sera, and all of them showed seroconversion in the convalescent sera (Fig. 4A and Table 4) with the OD values ranging from 0.534 to 2.775. The remaining four horses (#41–#44) had OD values over the cutoff value in the acute sera, although they also showed an obvious increase in OD values in the convalescent sera (Fig. 4B and Table 4) ranging from 1.353 to 3.000. When the antibody levels were expressed as titers by

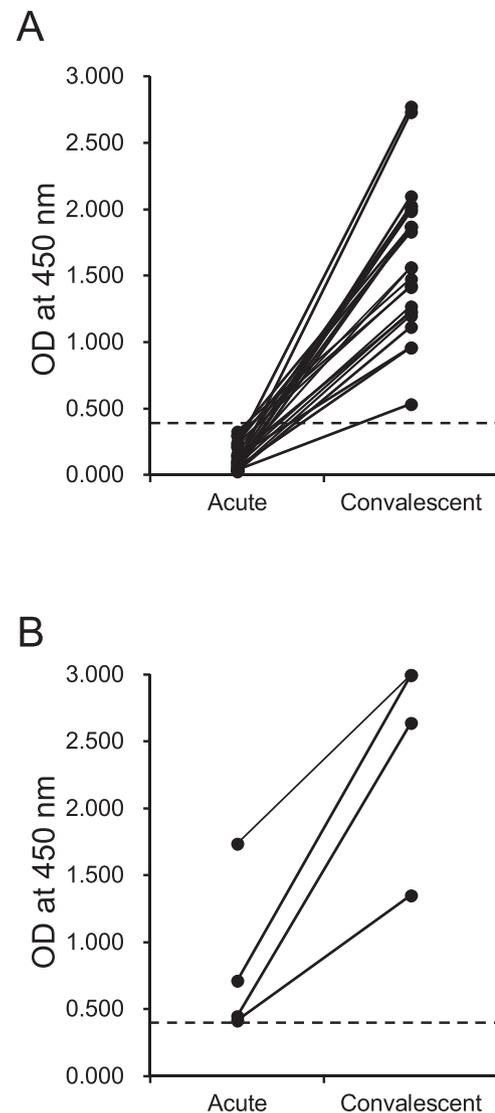


Fig. 4. rE2-ELISA for sera from horses naturally infected with Getah virus. (A) Paired sera from naturally infected horses ($n = 24$) whose first serum was seronegative. (B) Paired sera from naturally infected horses ($n = 4$) whose first serum was seropositive. Dashed line, cutoff value (0.352).

the endpoint method, these four horses showed a ≥ 4 -fold rise in titer between the paired sera (Table 4).

4. Discussion

The *Alphavirus* E2 is an envelope glycoprotein covering the viral surface together with the E1 protein. The E2 protein is reportedly highly immunogenic (Fong et al., 2014; Weger-Lucarelli et al., 2016), and several research groups have utilized antigenic epitopes of E2 to establish antibody detection methods for Chikungunya virus (Cho et al., 2008; Morey et al., 2010; Verma et al., 2014). Consistent with those reports, our current results showed the Getah virus-infected horse sera reacted strongly with viral proteins whose molecular weights corresponded to those of E1 and E2 proteins (Fig. 1A and B); further analysis using recombinant proteins suggested the rE2 protein was a promising candidate as an antigen for ELISA (Fig. 1C and D).

The ELISA established using rE2 protein successfully detected antibodies against Getah virus both in experimentally and naturally infected horses with a clear cutoff from negative controls (Figs. 3A and 4A, Tables 2 and 4). This method should be useful for serosurveillance of Getah virus infection among horse populations in a high-throughput

and time-saving way. Some of the naturally infected horses were shown in rE2-ELISA to be antibody-positive in the acute sera (Fig. 4B and Table 4), which may be attributable to previous exposure to the virus or to vaccination. In this case, even though the OD values increased between the paired sera, the definition of seroconversion in the single dilution method did not make sense, as the acute sera had OD values exceeding the cutoff value. The solution to this problem was to use the endpoint method which has been used in ELISAs for the diagnosis of equine rhinopneumonitis (Yasunaga et al., 1998; Bannai et al., 2016a), in which the majority of horses have pre-existing antibodies due to natural infection by the virus. With this method, an increase in the antibody titer by ≥ 4 -fold was successfully detected for the four horses (Table 4). As a result, rE2-ELISA could detect seroconversion of all naturally infected horses, either by the single dilution method or by the endpoint method. These results suggested that rE2-ELISA should be a good alternative to the VN test for the diagnosis of Getah virus infection. The three virus strains used in the experimental inoculation of horses were those isolated during the outbreaks in Japan (1978, 2014 and 2015). Although the rE2-ELISA detected seroconversion in all horses tested, there was a slight difference in the serum reactivities in the rE2-ELISA between horses inoculated with different strains (Table 2). However, it was unclear whether the difference could be attributable to the variation of virus strains, because the numbers of horses used in this study was too small to perform a statistical analysis. Also, further studies are required to address the variations in the reactivities of rE2-ELISA between Getah virus strains isolated in various regions worldwide.

As is often experienced with laboratory tests like ELISAs, the level of chemical reactions in each assay batch can be affected by laboratory conditions such as temperature and humidity, reagent lot, or even by the personnel performing the assays. In consideration of this point, the rE2-ELISA employed a system in which the cutoff value in each batch was corrected to the most appropriate value by using a standard curve drawn from serially diluted control serum (Fig. 2), which ensured the accuracy of diagnosis.

In Japan, where many horses are vaccinated with Getah virus vaccine, it would be ideal to establish a serological test with a capacity to differentiate infected from vaccinated animals (DIVA). The vaccine used in this study contains more than $10^{8.3}$ TCID₅₀/dose of formalin-inactivated Getah virus, and the antibody responses after vaccination was reported to be generally low: the VN titers after two doses of vaccination range 1:8 to 1:32 at the highest, and some horses even do not show detectable VN titers (less than 1:4) (Imagawa et al., 2003; Bannai et al., 2015). Our current result was consistent with these reports, and the low responses in VN titers were considered not due to a vaccine failure. Despite the low VN antibody responses, some horses vaccinated with Getah virus vaccine showed detectable levels of ELISA antibodies in their V2 and V2 + 28 sera (Fig. 3B and Table 3). The mean OD values of these sera were much lower than that shown in the experimentally infected horses; hence, vaccination may not greatly interfere with the interpretation of the ELISA results. However, some of the convalescent sera from naturally infected horses had OD values comparable to those of the vaccinated horses (Table 4), suggesting the rE2-ELISA does not have a DIVA capacity. This implies that, in horses vaccinated just before or soon after disease onset, seroconversion between the paired sera does not necessarily mean infection and might have occurred due to the vaccination. Therefore, the diagnosis of Getah virus infection using rE2-ELISA should be made in consideration of horses' vaccination history, and in some cases, virus isolation or detection of viral RNA from blood samples would be required for further confirmation. In terms of specificity, the rE2 protein showed reaction with the mouse ascitic fluid against RRV (Fig. 1E), although it is not clear whether sera from horses infected with RRV also cross react with rE2 protein. Thus, the use of rE2-ELISA should be limited in the geographical areas free from RRV to avoid misinterpretation of the test results.

In conclusion, we identified that Getah virus E2 protein was highly immunogenic, and rE2-ELISA detected horse antibodies against Getah virus after experimental and natural infections. Our study is the first to establish a recombinant protein-based ELISA for the detection of antibodies to Getah virus, which may be an alternative to the VN test used currently.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jviromet.2019.113681>.

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