

## Development and Evaluation of Indirect ELISA for Detection of Antibodies to Getah Virus in Horse Serum

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Getah virus (GETV) is a member of the genus *Alphavirus* in the family *Togaviridae*. GETV infection can occur in a wide range of vertebrate species, and the virus has been known for a pathogen of horses and pigs. To rapidly and accurately diagnose GETV infection of a racehorse, an indirect ELISA (I-ELISA) was developed in the present study for detection of antibodies to GETV in serum samples. To evaluate the developed I-ELISA, a total of 240 serum samples from Thoroughbred racehorses raised in Korea were screened in parallel by a serum neutralization (SN) test. The developed I-ELISA exhibited an efficacy comparable to that of the SN test in terms of a high diagnostic sensitivity (86.3%) and specificity (94.5%) at a cut-off absorbance value of 0.25. In addition, our results showed that the developed I-ELISA had a significant correlation with the SN test ( $r = 0.91$ ;  $p < 0.05$ ). Taken together, our findings suggest that the I-ELISA developed in this study is a valuable diagnostic tool for the screening of horses suspected to be infected with GETV.

**Key Words:** Getah virus, ELISA, Racehorse

### INTRODUCTION

Getah virus (GETV) is a member of the genus *Alphavirus* in the *Togaviridae*, was first isolated from mosquitoes (*Culex gelidus*) in Malaysia in 1955 (1). Based on antigenic properties, GETV is grouped into the Semliki Forest virus complex along with Ross River, Sagiya, Semliki Forest, Middleburg, Chikungunya, Barmah Forest, Bebaru, Mayaro, Una, and O'nyong-nyong viruses (2, 3). Serological studies of GETV suggest that the virus is widespread Eurasian and Australasian countries and among islands in the Pacific Ocean (4~7).

GETV infection has been identified in numerous verte-

brate species (8). GETV is maintained in nature by zoonotic transmission cycles involving nonhuman primate/vertebrate hosts and primary arthropod vectors (9). In these cycles, horses and pigs are amplifying hosts for GETV (10). Although horse-to-horse transmission is possible, mosquitoes (mainly *Culex* and *Aedes* species) are the most important arthropod vectors for GETV transmission to host animals (10~12). The GETV has been known for a pathogen of horses and pigs. The infection is associated with sporadic outbreaks of a mild febrile illness in horses, though it can also cause miscarriage in sow and death in newborn piglets (13, 14). Clinical symptoms in horse include fever, hind-limb edema and stiffness, but the disease is not life-threatening (15, 16). In addition, these clinical symptoms

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resemble other infectious and noninfectious diseases, including equine viral arteritis, equine rhinopneumonitis, equine encephalosis, equine influenza, equine infectious anemia, African horse sickness fever, *purpura haemorrhagica* and hoary alyssum toxicosis (17). Therefore, rapid and accurate diagnosis of GETV infection must be confirmed through laboratory examinations.

Previous serosurveillance studies reported large numbers of horses in endemic areas to be seropositive, despite the rarity of clinical symptoms associated with GETV infection. In Japan, 3~5% of horses have antibodies against GETV; the highest rate of seropositivity (93%) was found in older horses in northern Japan (15, 18, 19). In other countries, rates of seropositivity for GETV in horses have been reported to be 17% in India and 25% in Hong Kong (20, 21). In Korea, the first serosurveillance study of GETV was performed in 1987 from a total of 464 Thoroughbred racehorses (22). This study demonstrated that the rates of seropositivity for GETV in racehorses were 34%. According to the recent serosurveillance studies, the rates of seropositivity for GETV in racehorses were 12.4% and 12.2% in 2013 and 2014, respectively (22), significantly lower than that reported in 1987. These data suggest that due to an aggressive mosquito eradication program, the incidence of GETV infection has been decreased gradually, but the prevalence of viral infection in horses is likely to stay constant over the past two years in Korea.

Routine laboratory diagnosis of GETV infection is based on virus cultures and serology, followed by detection of the viral genome by reverse transcription-polymerase chain reaction (23, 24). However, these processes are time-consuming and labor-intensive, and the degree of success is dependent on a number of complicating factors (25). In general, rapid and specific detection is critical for the intensive surveillance of a viral disease. Serological assays such as hemagglutination inhibition and serum neutralization (SN) tests are considered the most specific methods for differentiating GETV from other antigenically related alphaviruses (12, 24, 26). ELISA is also considered to be a specific and sensitive method of detecting serum antibodies against GETV, equivalent to serological assays (27).

In the present study, we describe the development and evaluation of an indirect ELISA (I-ELISA) method for GETV antibodies and compare its specificity and sensitivity with those of the SN test.

## MATERIALS AND METHODS

### Serum samples, viruses and cells

The 240 serum samples analyzed in this study were obtained in 2013 from blood collected from Thoroughbred racehorses raised in Gyeonggi, Gyeongnam, Jeonbuk and Jeju, South Korea. The serum samples were heated at 56 °C for 30 min and stored at -20 °C until use. The racehorses were 1 to 23 years old. Approximately 70% of the racehorses were bred in Korea; the others were imported from various countries, including the United States of America, Australia and Japan. Most of the Korean racehorses did not receive a GETV vaccination; vaccination information was not available for racehorses imported from other countries. The QIAG9301 strain of GETV (NCBI accession number: KR-081238), isolated in 1993 from a swine blood sample (28), was provided by the Animal and Plant Quarantine Agency, Ministry of Agriculture, Food and Rural Affairs (MAFRA), Republic of Korea. The Vero cell line (ATCC CCL-81), from African green monkey kidney cells, was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA).

### Cell culture and virus propagation

The QIAG9301 strain of GETV was propagated in Vero cells. Vero cells were cultured in  $\alpha$ -MEM (Gibco, Grand Island, NY, USA), supplemented with 1% antibiotic-antimycotic (Gibco, Carlsbad, CA, USA), and 10% heat-inactivated fetal bovine serum (Life Technologies, Grand Island, NY). The cells were seeded into T-150 flasks at an initial density of  $1 \times 10^6$  and grown at 37 °C in a 5% CO<sub>2</sub> incubator. For virus infection, the cells at 80% confluency were rinsed twice with phosphate-buffered saline (PBS, pH 7.2) and then inoculated with the QIAG9301 strain. One hour post-infection, the cells were rinsed once with PBS and maintenance medium was added. The virus was harvested

by the freeze-thawing method when a GETV-specific cytopathic effect (CPE) was observed in the cells. To obtain cell-free viruses, the fluid was clarified by low-speed centrifugation (at  $3,000 \times g$  for 30 min). The supernatant was stored at  $-70^{\circ}\text{C}$  until use.

#### **Serum virus neutralization test**

Neutralizing antibodies to GETV were detected and quantified in 240 horse serum samples by using serum virus neutralization test (SN). SN tests were carried out in 96-well cell culture plates as described previously (29). All horse sera were inactivated at  $56^{\circ}\text{C}$  for 30 min and stored at  $-20^{\circ}\text{C}$  until use. The sera were diluted two-folds (1:2 to 1:256), and mixed with the equal volume of a virus suspension, containing approximately  $\sim 200 \text{ TCID}_{50}/0.1 \text{ ml}$  GETV. Mixture was incubated for 1 h at  $37^{\circ}\text{C}$ . After incubation for 1 h at  $37^{\circ}\text{C}$  in a  $\text{CO}_2$  incubator, a suspension of the Vero cells ( $2 \times 10^4$  / well) was added to each well. Plates were incubated at  $37^{\circ}\text{C}$  in an atmosphere with 5%  $\text{CO}_2$  for 3~4 days until the final reading. All samples were tested in duplicates and SN titers were established as reciprocal values of the highest serum dilutions resulting in the inhibition of cytopathic effect.

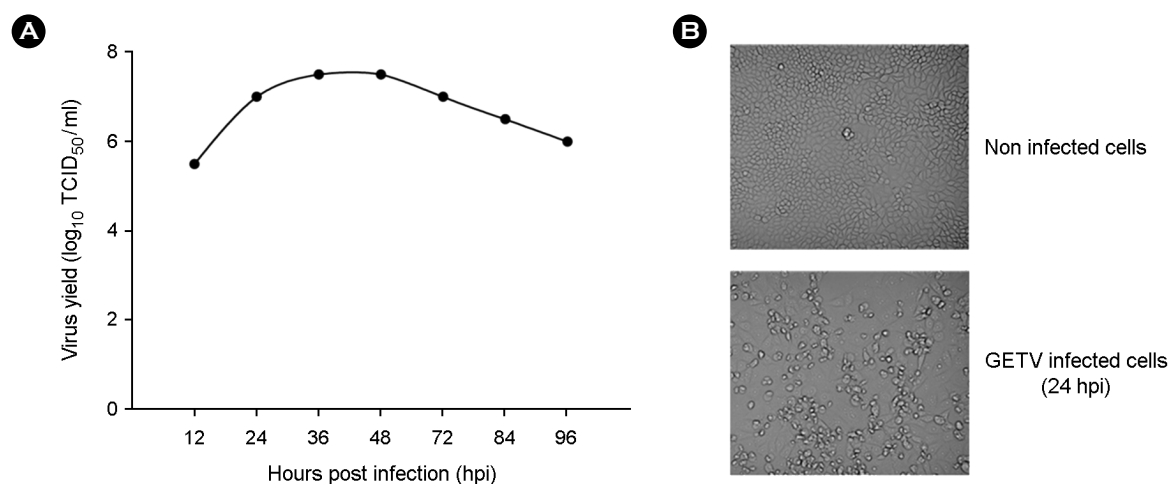
#### **Preparation of GETV antigen**

Viruses were concentrated as described previously (30, 31). The viral suspensions were filtered using membrane filters of  $0.2 \mu\text{m}$  pore size to remove unwanted cell debris and to produce clarified samples for further downstream purification. In brief, the pre-chilled viral suspension was subjected to precipitation using 10% (w/v) Polyethylene glycol 8000 (PEG 8000; Sigma-Aldrich) in the presence of 0.5 M (w/v) NaCl. The mixture was centrifuged at  $2,000 \times g$  for 10 min following overnight incubation at  $4^{\circ}\text{C}$ . The PEG-concentrated pellets were dissolved in GTNE buffer (200 mM glycine, 100 mM Tris-Cl, 100 mM NaCl and 1 mM EDTA, pH 7.6). The viruses were purified by discontinuous sucrose gradient centrifugation in ultracentrifuge. For this procedure, 20~60% (wt/wt) sucrose solution were prepared using GTNE buffer. The resuspended viruses were layered on the top of the 20% sucrose solution and centri-

fuged at  $100,000 \times g$  for 2 h. The clear band was collected carefully, and dialyzed against PBS to remove residual sucrose solution. Finally, the virus preparations were ultrafiltrated using Amicon ultracentrifugal filters (100 kDa) to increase the concentration of viruses in the sample, and then stored at  $-70^{\circ}\text{C}$  until use. The protein concentration was determined using a bicinchoninic protein assay kit (Pierce, Rockford, IL, USA).

#### **Indirect ELISA**

The optimum working concentrations of GETV antigen and serum for the I-ELISA were determined by the checkerboard titration method as described previously (29). In brief, 50  $\mu\text{l}$  of the GETV antigen, in an appropriate dilution ranging from 5 to 0.002  $\mu\text{g}/\text{ml}$  in 0.05 M carbonate-bicarbonate buffer (pH 9.6), were dispensed into a 96-well microtiter plate (Maxisorp; NUNC, Roskilde, Denmark). For antigen coating, the plate was incubated at  $4^{\circ}\text{C}$  overnight (at least 20 h). The wells were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBS-T), blocked with 5% skim milk for 2 h at  $37^{\circ}\text{C}$ , and then incubated with 100  $\mu\text{l}$  serially diluted serum samples (diluted in PBS-T from 20 to 40,960 folds) for 2 h at  $37^{\circ}\text{C}$ . Each test serum was tested in duplicate and the negative control sera and conjugate control were tested in triplicate. The wells were washed and incubated with 50  $\mu\text{l}$  horseradish peroxidase (HRP)-conjugated anti-horse IgG (KPL, Gaithersburg, MD, USA) for 1 h at  $37^{\circ}\text{C}$ . After washing, the color was developed using 50  $\mu\text{l}$  2,2'-azino-di-3-ethyl-benzthiazolinsulfonate (ABTS; KPL, Gaithersburg, MD, USA), and an equal volume of 1% (w/v) sodium dodecyl sulfate solution was used to stop the reaction. The absorbance at 405 nm was measured in a microplate spectrophotometer (Sunrise ELISA reader; Tecan, Switzerland). The results were expressed as the absorbance at 405 nm after subtracting the background value of the blank. Serum samples were evaluated as positive if the specific absorbance was higher than the cut-off absorbance value, which was calculated as the mean absorbance plus three times the standard deviation using negative serum samples in the SN test (SN titer of  $<1:2$ ). The specificity and sensitivity were calculated using the following formula:



**Figure 1.** The optimal time point for antigen harvest was determined by assessing GETV growth kinetics (A). The GETV-specific CPE was observed at ~24 hpi (B).

sensitivity (%) = (number of positives in both tests/total number of positives in the reference test)  $\times$  100; specificity (%) = (number of negatives in both tests/total number of negatives in the reference test)  $\times$  100.

### Statistical analysis

The correlation coefficients (*r* value) between the I-ELISA and SN tests were calculated using the linear regression program of GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA, USA). A *p* value < 0.05 was considered to indicate statistical significance.

## RESULTS

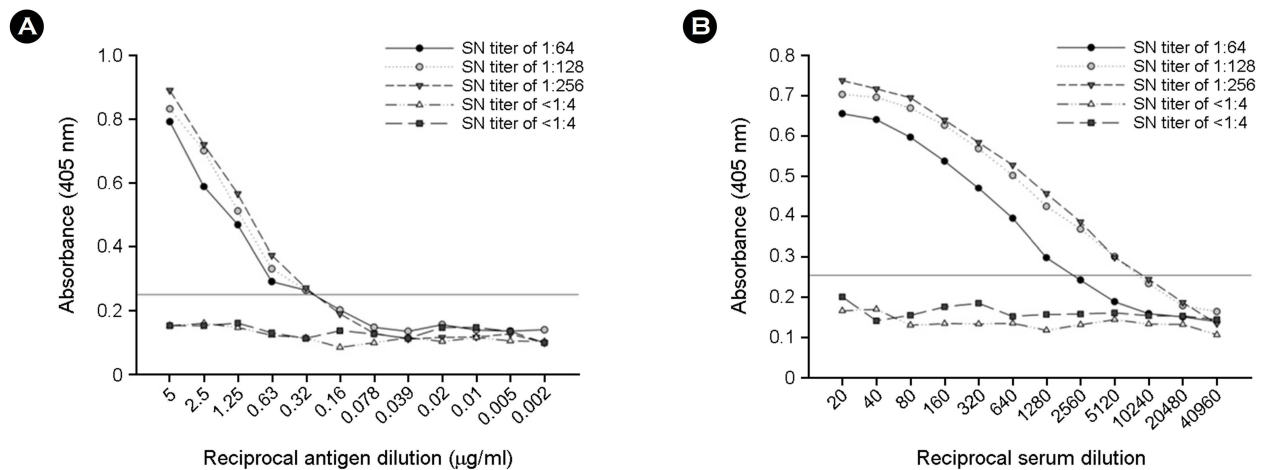
### Viral production

The optimal time for antigen harvest was determined based on the growth kinetics of GETV (Fig. 1A). Confluent Vero cells (CCL-81) grown in T25 flasks were incubated with 100 TCID<sub>50</sub>/0.1 ml GETV QIAG9301 strain for 1 h. After washing the Vero cells twice with PBS, maintenance medium was added, and the cultures were incubated at 37°C for various time intervals. The total amount of virus produced in each culture was quantified by virus titration every 12 h between 12 and 96 h post-infection (hpi). By 12 hpi, Vero cells infected with GETV produced an infectious

viral titer of 10<sup>5.5</sup> TCID<sub>50</sub>/ml. The viral titer increased exponentially between 12 and 24 hpi. During this time, the GETV-specific cytopathic changes, including vacuolization and condensation of the cytoplasm were observed in ~50% of monolayer of cells (Fig. 1B). A larger proportion of infectious progeny viruses appeared at 36 and 48 hpi, at a titer of 10<sup>7.5</sup> TCID<sub>50</sub>/ml. At 48 hpi, almost all cells were rounded and detaching from the surface of the flask. After 72 hpi, the viral titers declined gradually from 10<sup>7.0</sup> (72 hpi) to 10<sup>5.8</sup> TCID<sub>50</sub>/ml (96 hpi). Based on the GETV growth kinetics, viral antigen was harvested between 36 and 48 hpi. Harvested virus was purified by discontinuous sucrose gradient centrifugation. Purified whole GETV was concentrated to 1.09 mg/ml by ultrafiltration and then used as an antigen for I-ELISA.

### Determination of GETV-positive and -negative reference sera

The SN test was carried out on 240 horse serum samples to detect GETV-neutralizing antibodies. A total of 55 serum samples showing an SN titer of 1:64 or higher were used as positive reference sera. Negative reference serum samples were chosen from horse sera negative for GETV-neutralizing antibodies. A total of 150 serum samples showing a GETV neutralizing antibody titer of <1:2 were used as negative

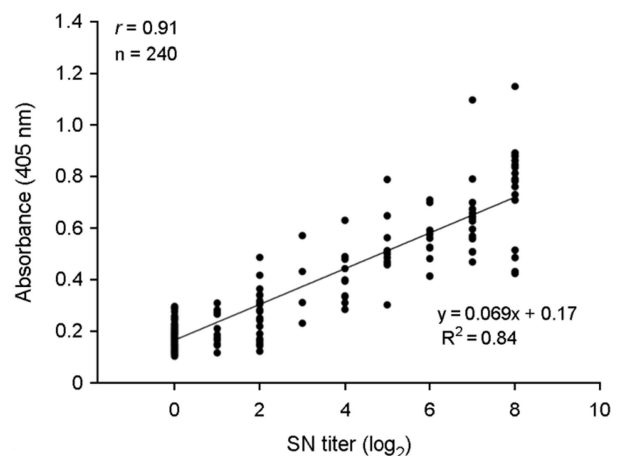


**Figure 2.** Checkerboard titration of horse serum against GETV antigen by I-ELISA. GETV-positive (SN titer of  $\geq 1:64$ ) and -negative (SN titer of  $< 1:2$ ) reference sera were determined by the SN test. According to the checkerboard titration, the optimum working concentration of GETV antigen (A) and dilution of reference sera (B) were 2.5 µg/ml and 1:40, respectively. The serum samples were evaluated as positive if their OD value was greater than 0.25. This OD cut-off value was calculated as noted in the Materials and Methods.

reference sera. These GETV-Positive and -Negative reference sera (P/N sera) were used for checkerboard titration.

### Development and optimization of I-ELISA

I-ELISA based on purified whole GETV antigen was developed for detection of GETV antibodies in serum samples from Thoroughbred racehorses. Optimum conditions for the I-ELISA were determined arbitrarily by means of checkerboard titration using the GETV-P/N sera (Fig. 2). The positive reference sera showed high absorbance values when cells were coated with 5 or 2.5 µg viral antigen. Therefore, 2.5 µg GETV antigen was used in subsequent experiments. The maximum difference between GETV-P/N sera was evident at a 1:40 dilution; therefore, this dilution was used in subsequent experiments. In addition, the maximum dilution of HRP-conjugated anti-horse IgG and the optimal reaction time were 1:4,000 and 1 h, respectively. In this test, a total of 150 serum samples that were negative according to the SN test (SN titer of  $< 1:2$ ) showed low reactivity to GETV antigen in the ELISA. These sera had an average absorbance of 0.17 with a standard deviation of 0.0392. Therefore, we set an absorbance value of 0.25 as the threshold (cut-off value) for the I-ELISA (horizontal line in Fig. 2). The most



**Figure 3.** Comparison of the SN test and I-ELISA for detection of GETV antibodies in 240 horse serum samples. The correlation between the two tests is indicated by the linear regression line and  $r$ -value.

suitable blocking buffer for the I-ELISA was 5% skim milk in PBS (data not shown).

### Comparison of the I-ELISA and SN test

To evaluate its diagnostic reliability, the I-ELISA was compared with the SN test using a total of 240 horse serum

**Table 1.** Determination of the sensitivity and specificity of the I-ELISA for detection of GETV antibodies in comparison with the SN test. The diagnostic sensitivity and specificity of the I-ELISA test were 86.3% and 94.5%, respectively<sup>a</sup>

		I-ELISA		
		Positive	Negative	Total
SN test	Positive	82	8	90
	Negative	13	137	150
	Total	95	145	240

Overall agreement 91.25%

<sup>a</sup>Relative specificity (%) =  $(137/145) \times 100 = 94.5\%$ . Relative sensitivity (%) =  $(82/95) \times 100 = 86.3\%$ .

samples. In Fig. 3, the correlation between the SN test and I-ELISA is indicated by the regression line and correlation coefficient  $r$ . The  $r$ -value was calculated using the linear regression program of GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA, USA) and was 0.91 ( $p < 0.05$ ). The diagnostic sensitivity and specificity of the I-ELISA test were calculated from known GETV-P/N sera using a traditional diagnostic assay, such as a two-sided contingency table. As shown in Table 1, 82 serum samples were positive and 137 negative by both the SN test and I-ELISA, which represented 91.25% of the total samples. In contrast, the two tests yielded different results for 21 serum samples. Among these, eight that were positive by the SN test were negative by I-ELISA, indicating a specificity of 94.5% for I-ELISA compared with the SN test. The 13 serum samples negative by the SN test were positive by I-ELISA, indicating a sensitivity of 86.3% for I-ELISA compared with the SN test.

## DISCUSSION

I-ELISA was developed to replace serological assays as a routine method for detection of GETV antibodies, because I-ELISA has several advantages, principally simplicity and rapidity. In the present study, we developed an I-ELISA for GETV antibodies in horse serum using purified whole GETV antigen. Viral antigens were prepared from Vero cell cultures

infected with GETV. To maximize the GETV antigen yield, the optimal time point for harvesting the virus was evaluated by analyzing the GETV growth kinetics (Fig. 1A). For this purpose, infectious viral titers were determined in culture supernatants collected at various time points post-infection. The maximum viral yield occurred between 36 and 48 hpi ( $10^{7.5}$  TCID<sub>50</sub>/ml); viral production decreased gradually thereafter. The GETV-specific CPE appeared at ~18 hpi (data not shown), and most of the cells showed cytopathic changes accompanying cell detachment at 48 hpi. Therefore, the cessation of viral production coincided with the maximum GETV-specific CPE. Therefore, GETV antigens were harvested between 36 and 48 hpi.

The diagnostic accuracy of the I-ELISA in terms of its sensitivity and specificity in comparison with those of the SN test was compared using a two-sided contingency table (Table 1). Eighty-two serum samples were positive, and 137 were negative in both tests. Therefore, the overall agreement between the two tests was 91.3%. An 86.3% sensitivity was achieved by testing 240 horse serum samples, of which 13 were negative by the SN test. The specificity was 94.5%; 8 serum samples positive by the SN test were negative by the I-ELISA. In addition, the results indicated a high correlation coefficient ( $r = 0.91$ ;  $p < 0.05$ ), suggesting a close relationship between the SN test and I-ELISA. Therefore, the developed I-ELISA has a reliability equivalent to that of the SN test. Taken together, these results suggested the developed I-ELISA to be a potentially useful diagnostic tool for screening samples suspected to harbor GETV.

Differences in the results for 21 of the samples were found between the two tests (Table 1). A total of 13 of 150 SN-negative samples were missed (8.7% false-positive rate), and 8 of 90 SN-positive samples were missed by I-ELISA (11.3% false-negative rate). Using the SN test as the "gold standard", the I-ELISA results for 8 of 90 SN-positive samples were erroneous. In contrast, the 13 of 150 SN-negative samples could be due to the different methods used for antibody detection between the two tests. I-ELISA detects total GETV-reactive antibodies, including those incapable of neutralization, whereas the SN test detects only neutralizing antibodies. A previous report on assays used to detect anti-

bodies against the bovine respiratory syncytial virus suggested a similar phenomenon (32).

The most important aspect of ELISA development is the antigen used, as this has a marked effect on the performance of the test (33). The whole GETV antigen used in this study was concentrated using PEG 8000 and purified by discontinuous sucrose gradient centrifugation. These methods resulted in a high yield of whole antigens with reduced non-specific activity. This simple process will facilitate large-scale production of whole GETV antigens.

In conclusion, this study describes the development of an I-ELISA for the rapid diagnosis of GETV infection in horses. The performance of the I-ELISA was evaluated in comparison with the SN test using a total of 240 horse serum samples. The high sensitivity and specificity of the I-ELISA suggested its potential for screening of horses suspected to be infected with GETV. The whole GETV antigen used for the I-ELISA was prepared by PEG precipitation and discontinuous sucrose gradient centrifugation of GETV antigen. This method enables whole GETV antigen preparation and development of I-ELISAs. Moreover, the I-ELISA developed in this study is economical due to the simple and rapid antigen preparation process.

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