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Development of a blocking ELISA for detection of Japanese encephalitis virus antibodies in pig and horse sera

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Abstract

Japanese encephalitis virus (JEV) is a mosquito-borne virus that can infect pigs, horses, and other mammals, including humans. Sero-epidemiological investigations of JEV have been performed using hemagglutination inhibition (HI), virus neutralization (VN) tests and enzyme-linked immunosorbent assay (ELISA). A need exists for a new ELISA that can detect JEV antibodies in the sera of several animal species. We aimed to develop a blocking ELISA (B-ELISA) for detecting JEV antibodies in pig and horse serum samples. JEV antibodies in 218 pig and 315 horse serum samples were measured using HI and VN tests. The purified KV1899-306 strain was used as an antigen for B-ELISA. The purified antibody (7A13) was conjugated with horseradish peroxidase and used as a detector antibody. The sera of pigs and horses to measure antibody against JEV were subjected to B-ELISA and analyzed. The B-ELISA had a diagnostic sensitivity of 94.6% to 100%, a specificity of 91.2 to 100%, and an accuracy of 94.9 to 98.6% compared with those of the HI and VN tests in pig and horse sera. The B-ELISA had a higher correlation with pig sera (r = 0.89 and 0.90 for VN and HI) than with horse sera (r = 0.75 and to 0.79). The new B-ELISA could be useful in the sero-surveillance of JEV in pig and horse sera and replace indirect ELISA.

Keywords: Japanese encephalitis virus; enzyme-linked immunosorbent assay; serology; viral zoonoses

Introduction

Japanese encephalitis (JE) is a zoonotic and mosquito-borne viral disease caused by the JE virus (JEV) belonging to the genus *Flavivirus* in the family *Flaviviridae*. Since the first clinical case of JE was reported in Japan in 1871, human JEV infections have been reported in 24 countries, including Australia and most Asian countries, such as China, Korea, and India [1–4]. In the Korean veterinary field, JEV has been identified in mosquitoes, pigs, and seals, since it was first isolated from pigs in 1969 [5,6]. The JEV genome encodes 3 structural proteins—the capsid, precursor membrane, and envelope E proteins—and 7 non-structural proteins—NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5. Among the 3 structural proteins, the E protein plays key roles in virulence and the induction of neutralizing antibodies (Abs) [7]. Although only one JEV serotype exists, on analyzing the nucleotide sequence of the genomic RNA, it is divided into 5 genotypes (G1–G5)

[8]. In South Korea, the genetic shift of JEV from G3 to G1 occurred in the late 1990s, and around 2010, G5 appeared and became the dominant genotype [5,6,9]. Although clinical signs of JEV infection in pigs serving as amplification hosts in the natural transmission of JEV are generally mild, JEV causes reproductive failure, such as abortion or still-birth in sows and fatal neurological disease in piglets [10]. Most horses infected with JEV are usually asymptomatic, but horses that develop clinical signs show fever, a decreased appetite, and neurological signs, such as circling and convulsion [11]. To prevent the resurgence of JE in animals in South Korea, annual serological monitoring has been performed using horse and pig serum. Other animals, including cattle, dogs, and goats, can be infected by mosquitoes carrying JEV [10]. The incidence and risk of JEV infection are increasing in several animals with global warming; however, serological studies to detect JEV Abs in several animal species are lacking in South Korea.

JEV Abs can be measured in serum samples through several methods. Hemagglutination inhibition (HI), the complement fixation test (CFT), virus neutralization (VN), the plaque reduction neutralization test (PRNT), and the enzyme-linked immunosorbent assay (ELISA) are standard methods for detecting JEV Abs in animals [12]. CFT is difficult to use because of its low sensitivity. Although the VN, PRNT, and HI tests are accurate and useful for measuring JEV Abs, they must be performed by skilled technicians and are unsuitable for large-scale serum screening. Therefore, a relatively simple assay, such as ELISA, is preferred to test a large number of sera samples. The general advantages of ELISA are that it does not require handling of live JEV in a biosafety cabinet, is fast and simple, and requires a small amount of serum [13]. Immunoglobulin (Ig) M-ELISAs have been developed for diagnosing recent JEV infections in humans and horses [14,15]. Blocking ELISA (B-ELISA) using the NS1 protein has been reportedly used to confirm field JEV infection in animals that were not vaccinated or inoculated with an inactivated vaccine [16]. In South Korea, where live JEV vaccine has been applied to animals, commercialized ELISA kits for serological monitoring of JE in several animal species are rare.

Previously, we reported that indirect ELISA (I-ELISA) had a sensitivity (91.8%–95.0%) and specificity (92.2%–94.7%) compared with those of the HI, VN, and PRNT tests [17]. The I-ELISA kit, which is economical and highly sensitive, was developed and commercialized to detect only JEV Abs in pig serum. In this study, we developed B-ELISA, which has the advantage of being applicable to sera from various animals because JEV transmitted by mosquitoes can cause disease in several animals. Since pigs and horses are continuously vaccinated in areas where JE is endemic, the use of B-ELISA will contribute to preventing JE infections by rapidly monitoring JE Abs.

Materials and Methods

Cells, viruses, and serum samples

C6/36 cells (*Aedes albopictus* clone, ATCC CRL-1660) were cultured in Dulbecco's modified Eagle's medium supplemented with 100 IU/mL penicillin, 10 μg/mL streptomycin, 0.25 μg/mL amphotericin B (Gibco BRL, USA), and 10% heat-inactivated fetal bovine serum (Gibco BRL) at 29°C in a CO_2 incubator. The C6/36 cells were used to propagate and titrate the KV1899-306 strain (KVCC no: VR2300018), which was passaged 306 times in various cells after isolation from pig sera. Vero cells (African green monkey kidney cells, ATCC CCL-81) were also used for an immunofluorescence assay (IFA) and the VN test. In total, 218 pig serum samples were collected from pig farms and 315 horse serum samples were collected from the Korea Racing Authority, for regular equine serum testing, in 2021. Whether the pigs or horses from which blood was collected were vaccinated against JE is unknown. All serum samples were subjected to VN and HI tests and B-ELISA. Positive serum samples for classical swine fever virus (CSFV), porcine respiratory reproductive syndrome virus (PRRSV), swine influenza virus (SIV), equine influenza virus (EIV) and West-Nile virus (WNV) included in commercialized ELISA kits were used for cross-reactivity.

Growth kinetics

The growth kinetics of the KV1899-306 strain was examined to determine the optimal harvest date for the C6/36 cells. Except for the use of different types of cells, the procedure was performed as previously described [18]. The viral titers of the KV1899-306 strain were determined in accordance with the standard method of Reed and Muench [19] and expressed as the 50% tissue culture infectious dose ($TCID_{50}$/mL).

Propagation and purification of the KV1899-306 strain

The C6/36 cells planted in 175 cm^2 cell culture flasks were infected with the KV1899-306 strain at a multiplicity of infection of 0.1. After incubation at 29°C for 4 days, the infected cells were frozen and thawed 3 times. JEV was purified using discontinuous sucrose gradient centrifugation, as described previously [17]. The purified JEV antigen concentration was determined 3 times using a NanoDrop 1000 UV/Vis spectrophotometer (Thermo Fisher Scientific, USA). Viral particles in the purified

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JEV were observed by electron microscope.

Characterization and conjugation of mAb 7A13

A monoclonal Ab (mAb; 7A13, Korean Collection for Type Cultures [KCTC] no: 18999P) against JEV was characterized using the indirect fluorescence assay, HI test, and western blot (WB) assay. For the IFA of 7A13, the Vero cells infected with the KV1899-306 strain were fixed, reacted with the mAb, and examined under a 100× fluorescence microscope (TE2000-U; Nikon Instruments, Japan). The HI test for 7A13 was performed according to the standard method [20]. For the WB assay, the purified JEV antigen was dissolved in sodium dodecyl sulfate-polyacrylamide gel sample buffer. The JEV antigen transferred to a nitrocellulose paper electrophoretically and reacted with the mAb (7A13). The blots were developed using the BCIP/NBT (Invitrogen, USA) substrate. The purified mAb 7A13 was conjugated with horseradish peroxide (HRP), as described previously [21]. The 7A13-HRP conjugate measured 3 times was used as the detector Ab for B-ELISA.

HI and VN tests

Before conducting the HI and VN tests, all serum samples were inactivated in a water bath at 56°C for 30 min. The HI and VN tests were performed as reported previously [17]. The HI titer of JEV was expressed as the reciprocal of the highest serum dilution that showed complete HI. An HI titer of 1:10 or higher was considered positive. The VN antibody (VNA) titer of JEV was expressed as the reciprocal of the highest serum dilution that completely inhibited the viral cytopathic effect (CPE). The serum was diluted 1:2 to 1:256. A VNA titer ≥ 1:2 was considered positive.

Optimization and application of B-ELISA

The checkerboard titration method was used to determine the optimal viral antigen concentration, serum dilution factor, and concentration of the 7A13-HRP conjugate. A 96-well microplate (Corning, USA) was coated with 100 μL of purified JEV antigen in carbonate buffer (pH 9.6) and incubated at 4°C overnight. After incubation, the solution was discarded, and the microplate coated with the JEV antigen was blocked with 1% casein solution. The purified JEV antigen at 3 concentrations (1 μg/mL, 2 μg/mL, and 4 μg/mL); serum panel samples at 3 dilutions (1:5, 1:10, and 1:20); and 7A13-HRP conjugate at 3 concentrations (0.5 μg/mL, 1 μg/mL and 1.5 μg/mL) were used to determine the optimal conditions for B-ELISA. The plate was washed with phosphate saline solution containing 0.05% Tween 20. After washing, 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB) solution and 50 μl of 0.5 M sulfuric acid were used as substrate and stop solutions, respectively. The absorbance at 450 nm was measured using an ELISA reader (Sunrise; Tecan, Switzerland). The percentage inhibition (PI) was calculated as follows: PI = (1 – absorbance value of the sample/absorbance value of the negative sample) × 100. The serum samples were evaluated as positive if the PI was > 40 in the pig serum and >30 in the horse serum. Calculations of specificity, sensitivity, and accuracy were performed using a previously reported method [17].

Statistical analysis

A linear regression analysis (least-squares method) was used to determine the correlation coefficient (r value) between the absorbance of B-ELISA and the HI or VNA titer. The r value was automatically calculated using the Excel ver. 2010 software (Microsoft Corp., USA).

Results

Propagation and purification of the KV1899-306 strain

The KV1899-306 strain exhibited unique CPEs (Fig. 1A) and was subjected to growth kinetics tests to determine the optimal harvest time. The viral content harvested daily for 7 days was determined in 96-well microplates. As shown in Fig. 1C, the KV1899-306 strain propagated in the C6/36 cells had the highest viral titer ($10^{7.0}$ $TCID_{50}$/mL) at 4 and 5 days post-inoculation (DPI). Therefore, the optimum harvest time for the KV1899-306 strain was determined to be 4 DPI. The JEV antigen of the KV1899-306 strain for the new B-ELISA was purified by sucrose gradient centrifugation after concentration with PEG-8000. The purified antigen containing 0.98 mg/mL was observed under an electron microscope, which revealed spherical viral particles 50 to 60 nm in diameter (Fig. 1D).

Characterization of the JEV-specific Ab

The 7A13 hybridoma secreting specific JEV Abs was propagated, and its activity was identified using the IFA, HI test, and WB assay. As shown in Fig. 2A–C, mAb 7A13 showed intracytoplasmic fluorescence in the JEV-infected Vero cells and had an HI titer of 1:20. In the WB assay, mAb 7A13 specifically reacted with the 54 k Da JEV E protein (Fig. 2D). The ascites fluid produced by BALB/c mice inoculated with 7A13 hybridoma was purified with a protein G column, and the concentration of the purified Ab was 2.6 mg/mL.

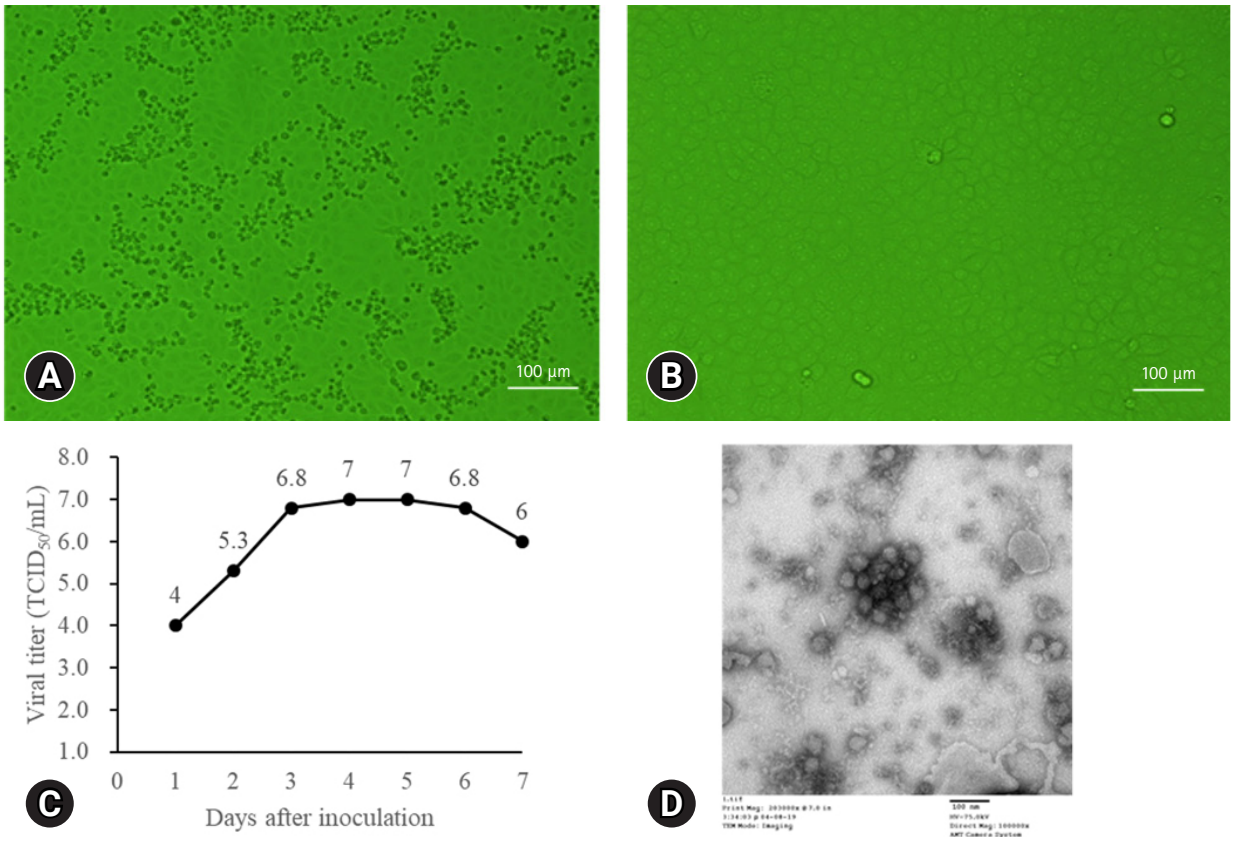

Fig. 1. Cytopathic effect of KV1899-306 strain that was infected into C6/36 cells (A) and normal cells (B), growth kinetics of KV1899-306 strain (C) and virus particles of KV1899-306 strain (D). Scale bars: (A, B) 100 μm.

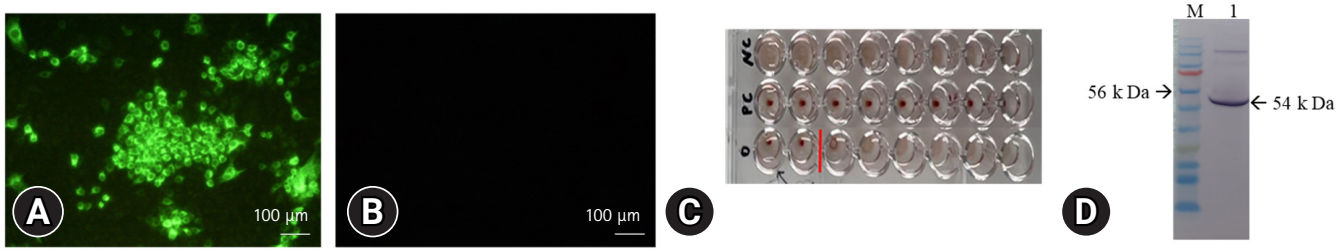

Fig. 2. Confirmation of Japanese encephalitis (JEV)-specific monoclonal antibody 7A13 using immunofluorescence assay (A, B), hemagglutination inhibition (HI) (C), and western blot assay (D). Scale bars: (A, B) 100 μm. M, protein ladder; lane 1, purified JEV antigen. Monoclonal antibody 7A13 shows specific intracytoplasmic fluorescence in the Vero cells infected with JEV KV1899-306 strain and has a HI titer of 1:20. In the western blot assay, it reacts to the JEV E protein (54 k Da).

Optimization of B-ELISA

The JEV antigen was tested at 3 concentrations to determine the optimal coating concentration. As shown in Fig. 3A, the 2 μg/mL JEV antigen had the highest negative serum/positive serum (N/P) value (1.29). The serum samples diluted to 1:5, 1:10, and 1:20 were tested to determine the optimal serum dilution rate; 1:10 had the highest N/P value (2.23) and was selected as the serum dilution rate (Fig. 3B). The 7A13-HRP conjugate concentration had the highest N/P value (1.69) at 1 μg/mL among the 3 concentrations (Fig. 3C).

Correlation and application of the serologic assay

The absorbance value from B-ELISA was compared with those of the titers of VN and HI using 218 pig serum samples and 315 horse serum samples to evaluate its diagnostic reliability. In Figs. 4 and 5, correlations between the PI% of B-ELISA

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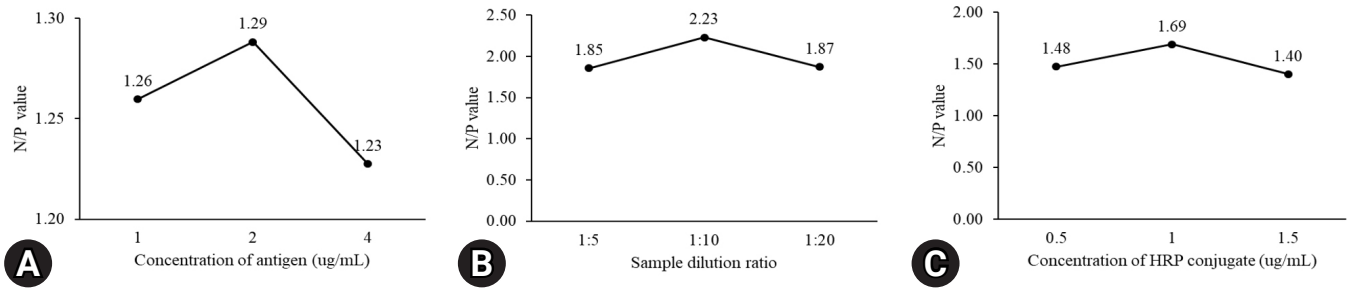

Fig. 3. Determination of the purified Japanese encephalitis (JEV) antigen concentration (A), serum dilution factor (B) and 7A13-horseradish peroxidase (HRP) conjugate concentration (C) for establishing blocking enzyme-linked immunosorbent assay (ELISA) on the basis of the negative serum/positive serum (N/P) absorbance value using the horse sera. The highest N/P values were used to determine the optimal antigen concentration (2.0 μg/mL), serum dilution (1:10), and 7A13-HRP conjugate concentration (1 μg/mL) for the blocking ELISA.

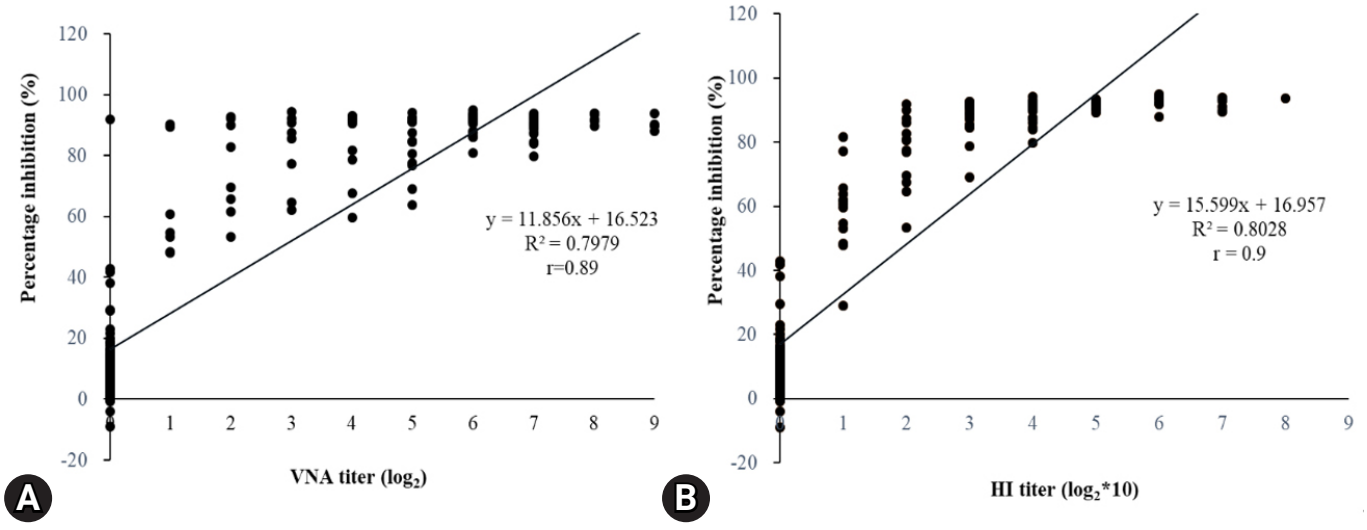

Fig. 4. Correlation between the VNA (A) and hemagglutination inhibition (HI) (B) titer and percent inhibition of blocking enzyme-linked immunosorbent assay (ELISA) for detecting Japanese encephalitis (JEV) antibodies using 218 pig serum samples. The r values of virus neutralization (VN) and HI in the pig sera were 0.89 and 0.9, respectively.

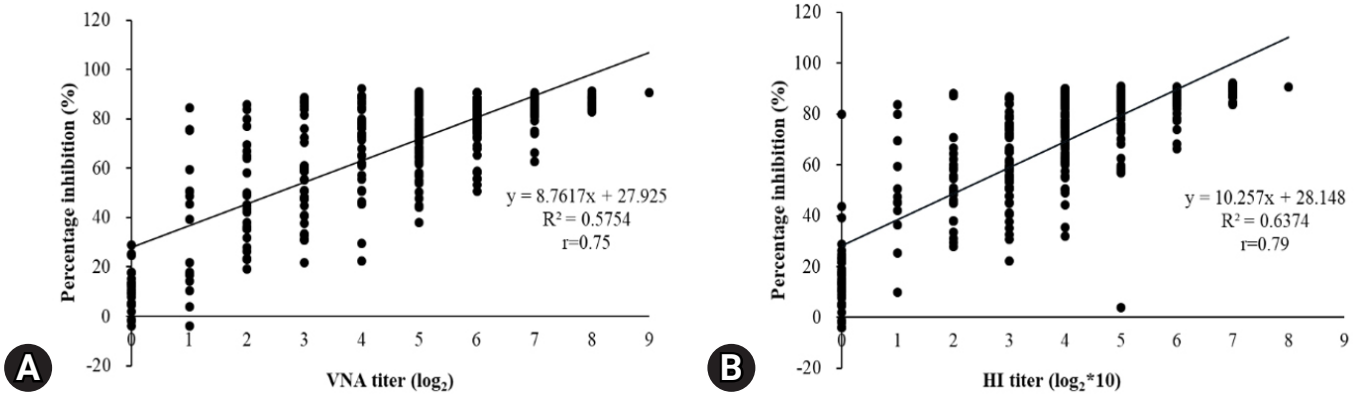

Fig. 5. Correlation between the virus neutralization antibody (VNA) (A) and hemagglutination inhibition (HI) (B) titer and percent inhibition of blocking enzyme-linked immunosorbent assay (ELISA) for detecting Japanese encephalitis (JEV) antibodies using 315 horse serum samples. The r values of virus neutralization (VN) and HI in horse sera were 0.75 and 0.79, respectively.

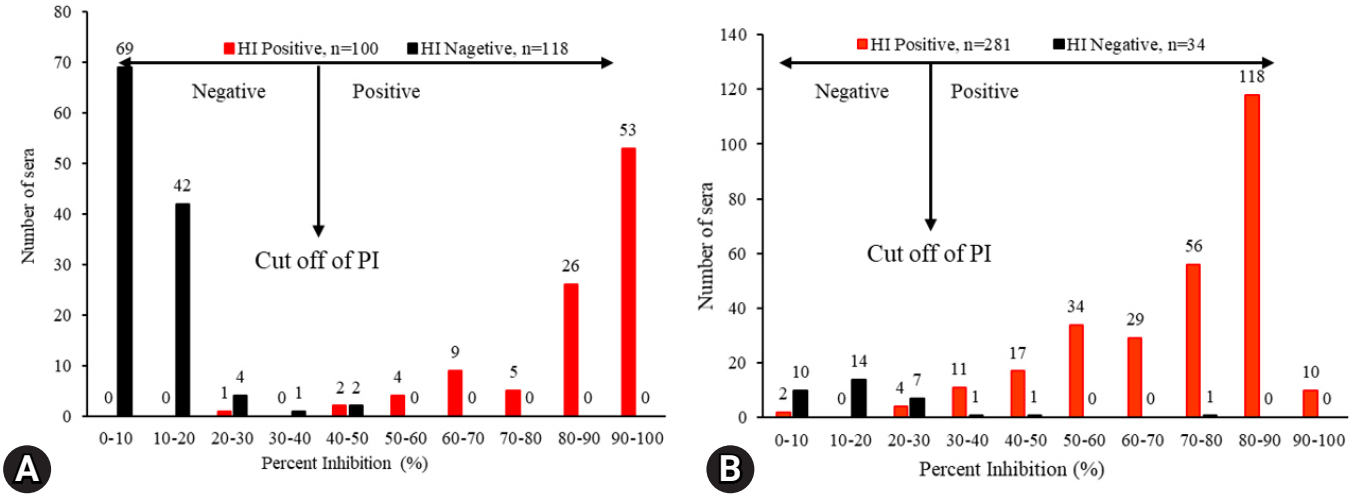

Fig. 6. Distribution of percentage inhibition (PI) values obtained by blocking enzyme-linked immunosorbent assay (ELISA) for pig sera (n = 218) and horse sera (n = 315). The arrows indicate cutoff values of 40 for pig sera and 30 for horse sera. Of the pig serum samples, 99.0% (99/100) of positive serum samples for Japanese encephalitis virus (JEV) had a PI value of > 40 (A), and of the horse serum samples, 97.9% (275/281) of positive serum samples had a PI value of > 30 (B).

and VN and between the PI% of B-ELISA and HI are indicated by regression lines. The r values for VN and HI were 0.89 and 0.9 in the pig sera and 0.75 and 0.79 in horse sera, respectively. As shown in Fig. 6, 99 (99.0%) of 100 pig serum samples showing an HI titer of 1:10 or higher had PI values ≥ 40, and 275 (97.9%) out of 281 horse serum samples with an HI titer of 1:10 or higher had PI values ≥ 30. The B-ELISA results were positive if the PI of the pig and horse serum samples exceeded 40% and 30%, respectively, and were negative if they were less than 40% and 30%, respectively (Fig. 6). The sensitivity, specificity, and accuracy of B-ELISA using 218 pig serum samples were 100%, 97.5%, and 98.6%, respectively, compared with those of the VN test, and 99.0%, 98.3%, and 98.6%, respectively, compared with those of the HI test (Table 1). The sensitivity, specificity, and accuracy of B-ELISA using 315 horse serum samples were 94.6%, 100.0%, and 94.9%, respectively, compared with those of the VN test, and 97.9 %, 91.2 %, and 97.1%, respectively, compared with those of the HI test (Table 1).

Cross-reactivity in the B-ELISA

The B-ELISA was developed with the specific JEV mAbs, and specificity of the B-ELISA was examined with 5 positive serum samples for CSFV, PRRSV, SIV, EIV and WNV. As shown in Table 2, the PI value of the sera for the CSFV, PRRSV, SIV, EIV was 5 or less, indicating negative, whole, the PI value of the horse serum for WNV was 51.1, indicating positive.

Discussion

JEV transmitted by the *Culex* mosquito causes diseases in several animal species. Pigs serving as the amplifying host of JEV are related to spillover to humans. Horses, the end hosts of JEV, can develop fatal encephalitis upon infection. Cattle infected with JEV develop neutralizing Abs [22]. JEV infection in various animal species, such as goats, dogs, and cats, is mostly subclinical and induces HI or neutralizing Abs [11]. Ardeid wading birds are involved in enzootic transmission. JEV infection is regionally extending from Asia to southern Australia, Angola, and Italy [23]. Although JEV infection has not been reported in livestock in Korea since 2008, JE incidence in humans is increasing every year [24]. Vaccination against JE in pigs and horses is believed to have contributed to the non-occurrence of JE. Approximately half (49.7%) of the Korean horses in 2007 were seropositive for JEV [25]. Although JE has been monitored annually in pig and horse sera, commercialized ELISA kits for horse sera are unavailable in South Korea. In the event JE re-emerges owing to climate change, the demand for serological tests for JEV will increase. Therefore, the development of a B-ELISA to measure JEV-specific Abs in pigs and horses would be timely.

The VN test for measuring anti-JEV Abs requires skilled technicians and cell culture facilities, including microscopes and CO_2 incubators. The HI test also requires goose blood cells and is a slightly complicated procedure. While, VN and HI tests are the standard methods for JEV serological studies, many lab-

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Table 1. Sensitivity, specificity, and accuracy of the blocking ELISA to detect anti-JEV antibodies in comparison with the VN and HI tests using pig and horse sera

	VN			HI		
	Positive	Negative	Total	Positive	Negative	Total
Pig sera						
Blocking ELISA						
Positive	98	3	101	99	2	101
Negative	0	117	117	1	116	117
Total	98	120	218	100	118	218
Sensitivity	98/98 (100.0)			99/100 (99.0)		
Specificity	117/120 (97.5)			116/118 (98.3)		
Accuracy	(98+117)/218 (98.6)			(99+116)/218 (98.6)		
Horse sera						
Blocking ELISA						
Positive	278	0	278	275	3	278
Negative	16	21	37	6	31	37
Total	294	21	315	281	34	315
Sensitivity	278/294 (94.6)			275/281 (97.9)		
Specificity	21/21 (100.0)			31/34 (91.2)		
Accuracy	(278+21)/315 (94.9)			(275+31)/315 (97.1)		

Values are presented as number only or number (%). Sensitivity (%) = [(no. of positives in both tests) / (no. of positives in the VN or HI test)] × 100. Specificity (%) = [(no. of negatives in both tests) / (no. of negatives in the VN or HI test)] × 100. Accuracy (%) = [(no. of positives in both tests + no. of negatives in both tests) / (total number of samples)] × 100.
ELISA, enzyme-linked immunosorbent assay; JEV, Japanese encephalitis; VN, virus neutralization; HI, hemagglutination inhibition.

Table 2. Cross-reaction of 5 positive serum samples in B-ELISA

Serum samples	Host	PI (%)	Result
Antibody to CSFV	Swine	3.2	Negative
Antibody to PRRSV	Swine	1.5	Negative
Antibody to SIV	Swine	3.4	Negative
Antibody to EIV	Equine	4.6	Negative
Antibody to West-Nile virus	Equine	51.1	Positive

B-ELISA, blocking enzyme-linked immunosorbent assay; PI, percentage inhibition; CSFV, classical swine fever virus; PRRSV, porcine respiratory reproductive syndrome virus; SIV, swine influenza virus; EIV, equine influenza virus.

oratories prefer ELISAs, which are easy to perform without specialized equipment. The previously developed I-ELISA is limited to pig serum [17]. Therefore, we developed a new B-ELISA using a purified antigen and mAb to measure JEV-specific Abs in the sera of several animals.

Several ELISAs have been reported to measure anti-JEV Abs in humans and animals [14–16]. An IgM ELISA was developed for diagnosing recent JEV infections. A B-ELISA using the NS1 protein was developed to differentiate between vaccination and field infection with serum, whereas an I-ELISA developed using purified JEV can be applied to animals bitten by mosquitoes carrying JEV. However, these ELISAs cannot be applied for the serological monitoring of JEV in various animals. This can be overcome by using B-ELISA that utilizes the competition between the Ab in serum and HRP-conjugated Ab [13]. In our B-ELISA, mAb 7A13 conjugated with peroxidase reacted with a coated virus that was not blocked by the JEV Abs in the serum. When the TMB substrate was added to the B-ELISA plate, a decrease in color intensity indicated the presence of anti-JEV Abs in the tested serum. Considering that Abs against JEV have been confirmed in pigs, horses, cattle, and goats [10], developing a tool for the sero-surveillance of several animals is worthwhile.

The sensitivity, specificity, and accuracy of B-ELISA were analyzed on the basis of the results of the VN and HI tests using 218 pig and 315 horse serum samples. The sensitivities (94.6%–100%) of B-ELISA in pig and horse sera compared with those of the VN and HI tests were higher than those of previous studies [17,26]. The specificities (91.2%–100%) of B-ELISA were similar or higher than those of the I-ELISAs using purified viral particles (91.8%–98.0%) [17,26]. The sensitivity and specificity of an ELISA can be enhanced using an mAb that blocks the epitope [27]. In our study, we used mAb 7A13, which has neutralizing activity against JEV, as the detector Ab. The properties of mAb 7A13 affected the sensitivity and specificity of B-ELISA. Moreover, B-ELISA showed high accuracy (94.9%–98.6%) in pig and horse sera, indicating that it is suitable for the sero-surveillance of JEV in pigs and horses.

Reproducibility, precision and stability of the B-ELISA kit will be mentioned in the documents required for the industrializa-

tion process. Further serological investigations using B-ELISA in sera from other animals, including cattle, are required.

In conclusion, this study describes the development of a new B-ELISA for measuring JEV Abs in pig and horse sera. The performance of B-ELISA was compared with those of the VN and HI tests using 218 pig and 315 horse serum samples. The high sensitivity, specificity, and accuracy of B-ELISA suggested its potential in monitoring pigs and horses suspected of being infected with JEV or for measuring the immune response after vaccination. Furthermore, this new B-ELISA can be used to measure JEV Abs in serum samples from various other animals, including cattle and goats.

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Author's Contributions

Conceptualization: Yang DK; Data curation: Yang DK, Kim EJ, Jang SH; Formal analysis: Yang DK, Kim EJ; Funding acquisition: Yang DK, Cho YS; Investigation: Yang DK; Methodology: Kim EJ, Jang SH, Kim B, Lee JA; Project administration: Yang DK; Software: Yang DK, Kim EJ; Validation: Kim EJ, Jang SH; Writing–original draft: Yang DK; Writing–review & editing: all authors.

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References

1. Erlanger TE, Weiss S, Keiser J, Utzinger J, Wiedenmayer K. Past, present, and future of Japanese encephalitis. *Emerg Infect Dis*. 2009;15:1–7.
2. Zheng Y, Li M, Wang H, Liang G. Japanese encephalitis and Japanese encephalitis virus in mainland China. *Rev Med Virol*. 2012;22:301–322.
3. Rajaiah P, Kumar A. Japanese encephalitis virus in India: an update on virus genotypes. *Indian J Med Res*. 2022;156:588–597.
4. Pham D, Howard-Jones AR, Hueston L, Jeoffreys N, Doggett S, Rockett RJ, et al. Emergence of Japanese encephalitis in Australia: a diagnostic perspective. *Pathology*. 2022;54:669–677.
5. Sanborn MA, Wuertz KM, Kim HC, Yang Y, Li T, Pollett SD, et al. Metagenomic analysis reveals Culex mosquito virome diversity and Japanese encephalitis genotype V in the Republic of Korea. *Mol Ecol*. 2021;30:5470–5487.
6. Yang DK, Kim BH, Kweon CH, Kwon JH, Lim SI, Han HR. Biophysical characterization of Japanese encephalitis virus (KV1899) isolated from pigs in Korea. *J Vet Sci*. 2004;5:125–130.
7. Villordo SM, Gamarnik AV. Genome cyclization as strategy for flavivirus RNA replication. *Virus Res*. 2009;139:230–239.
8. Solomon T, Ni H, Beasley DW, Ekkelenkamp M, Cardosa MJ, Barrett AD. Origin and evolution of Japanese encephalitis virus in southeast Asia. *J Virol*. 2003;77:3091–3098.
9. Jung JY, Kim HJ, Lee K, Choi JG, Kim YH, Lee KK, et al. Co-infection of Dirofilaria immitis and Japanese encephalitis virus in a spotted seal (Phoca largha) in the Republic of Korea. *J Vet Sci*. 2019;20:e65.
10. Mansfield KL, Hernández-Triana LM, Banyard AC, Fooks AR, Johnson N. Japanese encephalitis virus infection, diagnosis and control in domestic animals. *Vet Microbiol*. 2017;201:85–92.
11. Morita K, Nabeshima T, Buerano CC. Japanese encephalitis. *Rev Sci Tech*. 2015;34:441–452.
12. Roberts A, Gandhi S. Japanese encephalitis virus: a review on emerging diagnostic techniques. *Front Biosci (Landmark Ed)*. 2020;25:1875–1893.
13. Sakamoto S, Putalun W, Vimolmangkang S, Phoolcharoen W, Shoyama Y, Tanaka H, et al. Enzyme-linked immunosorbent assay for the quantitative/qualitative analysis of plant secondary metabolites. *J Nat Med*. 2018;72:32–42.
14. Chow L, Yueh YY, Hwang YS, Lin TL, Wu YC, Horng CB. Detection of IgM antibody to Japanese encephalitis virus infection by enzyme-linked immunosorbent assay (ELISA). *Zhonghua Yi Xue Za Zhi (Taipei)*. 1996;58:1–6.
15. Sahu A, Dhanze H, Singh V, Mehta D, Gupta M, Singh M, et al. Development of IgM-ELISA for diagnosis of recent infection of Japanese encephalitis virus in equines. *Biologicals*. 2022;75:16–20.

https://doi.org/10.14405/kjvr.20240024

16. Zhou D, Pei C, Yang K, Ye J, Wan S, Li Q, et al. Development and application of a monoclonal-antibody-based blocking ELISA for detection of Japanese encephalitis virus NS1 antibodies in swine. *Arch Virol*. 2019;164:1535–1542.
17. Yang DK, Kim HH, Jo HY, Lee SH, Jang SH, Lee SO, et al. Improvement of indirect enzyme-linked immunosorbent assay for detection of Japanese encephalitis virus antibodies in swine sera. *Korean J Vet Res*. 2017;57:31–36.
18. Lee SH, Yang DK, Kim HH, Jo HY, Choi SS, Cho IS. Development and evaluation of indirect ELISA for detection of antibodies to Getah virus in horse serum. *J Bacteriol Virol*. 2016;46:63–70.
19. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. *Am J Hyg*. 1938;27:493–497.
20. Clarke DH, Casals J. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *Am J Trop Med Hyg*. 1958;7:561–573.
21. Nakane PK, Kawaoi A. Peroxidase-labeled antibody. A new method of conjugation. *J Histochem Cytochem*. 1974;22:1084–1091.
22. Ilkal MA, Dhanda V, Rao BU, George S, Mishra AC, Prasanna Y, et al. Absence of viraemia in cattle after experimental infection with Japanese encephalitis virus. *Trans R Soc Trop Med Hyg*. 1988;82:628–631.
23. Mackenzie JS, Williams DT, van den Hurk AF, Smith DW, Currie BJ. Japanese encephalitis virus: the emergence of genotype IV in Australia and its potential endemicity. *Viruses*. 2022;14:2480.
24. Lee AR, Song JM, Seo SU. Emerging Japanese encephalitis virus genotype V in Republic of Korea. *J Microbiol Biotechnol*. 2022;32:955–959.
25. Yang DK, Kim BH, Kweon CH, Nah JJ, Kim HJ, Lee KW, et al. Serosurveillance for Japanese encephalitis, Akabane, and Aino viruses for Thoroughbred horses in Korea. *J Vet Sci*. 2008;9:381–385.
26. Shimoda H, Inthong N, Noguchi K, Terada Y, Nagao Y, Shimojima M, et al. Development and application of an indirect enzyme-linked immunosorbent assay for serological survey of Japanese encephalitis virus infection in dogs. *J Virol Methods*. 2013;187:85–89.
27. Goh LY, Kam YW, Metz SW, Hobson-Peters J, Prow NA, McCarthy S, et al. A sensitive epitope-blocking ELISA for the detection of Chikungunya virus-specific antibodies in patients. *J Virol Methods*. 2015;222:55–61.