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Comparison of JEV Neutralization Assay Using Pseudotyped JEV with the Conventional Plaque-Reduction Neutralization Test

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We previously reported the development of a neutralization assay system for evaluating Japanese Encephalitis Virus (JEV) neutralizing antibody (NAb) using pseudotyped-JEV (JEV-PV). JEV-PV-based neutralization assay offers several advantages compared with the current standard plaque-reduction neutralization test (PRNT), including simplicity, safety, and speed. To evaluate the suitability of the JEV-PV assay as new replacement neutralization assay, we compared its repeatability, reproducibility, specificity, and correlated its results with those obtained using the PRNT. These analyses showed a close correlation between the results obtained with the JEV-PV assay and the PRNT, using the 50% plaque reduction method as a standard for measuring NAb titers to JEV. The validation results m*et al*l analytical acceptance criteria. These results suggest that the JEV-PV assay could serve as a safe and simple method for measuring NAb titer against JEV and could be used as an alternative approach for assaying the potency of JEV neutralization.

***Keywords*:** Japanese encephalitis virus, neutralizing antibody, pseudovirus, PRNT

Introduction

Japanese encephalitis virus (JEV) is a serious mosquito-borne flavivirus that occurs throughout most of Asia and parts of the western Pacific (Solomon *et al.*, 2000). Among the estimated 35,000–50,000 annual cases, approximately 20–30% of patients die and 30–50% of survivors have neurologic or psychiatric sequelae (Solomon, 2003; Fischer *et al.*, 2010). JEV vaccines are usually made with Nakayama-NIH, Beijing-1, or SA14-14-2 strains. Formalin-inactivated vaccines are produced from the prototype Nakayama and Beijing-1 strains, and a live attenuated vaccine is produced from SA14-14-2. Great strides have been made in recent years toward controlling JEV, spurred by improved and expanded surveillance in endemic countries, increased availability of safe and effective JEV vaccines, and introduction of JEV immunization programs in several countries (Elias *et al.*, 2009; Halstead and Thomas, 2010). At present, vaccination and surveillance of JEV antibodies in humans are considered very important public health measures (Abe *et al.*, 2003; Jmor *et al.*, 2008).

Methods for measuring antibodies against JEV include hemagglutination inhibition (HI) tests, complement-fixation tests, neutralization tests and enzyme-linked immunosorbent assays (ELISAs), among others (Lee *et al.*, 1999; Markoff, 2000; Konishi *et al.*, 2004; Yang *et al.*, 2006; Biswas *et al.*, 2009; Ahsan and Gore, 2011). A new approach for measuring virus-neutralizing antibodies dispenses with the virus completely and uses instead an "infectious" vector that expresses viral E protein or E/prM proteins, with or without genes that encode "reporter" molecules (Pierson *et al.*, 2006). These assays are based on the observation that essentially all virus-neutralizing antibodies are elicited by the E protein. Among serological tests available for assessing specific host immune responses, the neutralization test is considered the most sensitive and specific for evaluation of vaccine efficacy. This test is based on the principle that infectious virus can be neutralized by specific antibodies, resulting in a loss of the ability to infect permissive cells (Simoes *et al.*, 2012).

The plaque reduction neutralization test (PRNT) is a specific serological test used to identify JEV infection in diagnostic laboratories and to evaluate the neutralizing antibody (NAbs) response of vaccines. The PRNT is the 'gold standard' for detecting and quantifying JEV NAbs (Shyu *et al.*, 1997; Ting *et al.*, 2001; Roehrig *et al.*, 2008). The NAb test using chick embryo (CE) cells or Vero cells has been adopted for the assessment of antibody in human serum (Abe *et al.*, 2003), and the World Health Organization (WHO) recommends the use of BHK-21 cells for quality testing of JEV vaccines (e.g., using the virus-inactivation test), as well as for estimating potency using the PRNT assay (Hombach *et al.*, 2005).

PRNTs of JEV utilize live, biohazardous JEVs, and require a cell culture facility, considerable technical expertise in growing and titrating JEV plaques, and an appropriate biosafety level laboratory (BSL3) (Johnson *et al.*, 2009; Lobigs *et al.*, 2010). In order to overcome these shortcomings, we constructed a pseudotyped murine leukemia virus encoding JEV (Nakayama-NIH) E protein and β-galactosidase (Lee *et al.*,

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Table 1. Neutralizing antibody titers using PRNT or PV assay

Sample no.	PRNT		PVs assay	
	NAb titer	NAb titer (log)	NAb titer	NAb titer (log)
1	859	2.93	931	2.97
2	1,000	3.00	928	2.97
3	1,243	3.09	905	2.96
4	315	2.50	435	2.64
5	1,175	3.07	1,280	3.11
6	629	2.80	1,280	3.11
7	1,280	3.11	1,056	3.02
8	157	2.20	458	2.66
9	839	2.92	1,011	3.00
10	1,254	3.10	1,280	3.11
11	534	2.73	1,280	3.11
12	279	2.45	358	2.55
13	1,186	3.07	867	2.94
14	158	2.20	269	2.43
15	1,262	3.10	1,280	3.11
16	156	2.19	165	2.22
17	1,241	3.09	1,280	3.11
18	1,239	3.09	1,137	3.06
19	311	2.49	457	2.66
20	1,280	3.11	1,280	3.11
21	1,075	3.03	840	2.92
22	1,280	3.11	1,280	3.11
23	299	2.48	321	2.51
24	1,253	3.10	1,280	3.11
25	136	2.13	308	2.49
26	1,280	3.11	1,280	3.11
27	318	2.50	260	2.41
28	1,280	3.11	1,116	3.05
29	10	1.00	48	1.68
30	10	1.00	51	1.71

2009). Using this pseudotyped virus (PV), we developed a simple, rapid, and safe neutralization assay system (JEV-PV) in Vero cells. To evaluate the suitability of the JEV-PV assay as new replacement neutralization assay, we validated its repeatability, reproducibility, and correlation with the PRNT.

Materials and Methods

Cells

TELCeb6 cells and Vero cells (CCL-81; American Type Culture Collection [ATCC]) were maintained in Dulbecco's Modified Eagle's medium (DMEM; Thermo Scientific, USA) containing 10% fetal bovine serum (FBS; Thermo Scientific) and 1% penicillin/streptomycin (Invitrogen, USA).

Serum samples

The Serum samples were collected from human volunteers after obtaining informed consent. Whether the antibodies originated from vaccination or natural infection was not known. Sera used in this study, shown in Table 1, were obtained from the Center for Immunology and Pathology, Korea National Institute of Health. All the samples used during this study were obtained following written informed consent from the donors and performed with the approval of the IRB at Inha University Hospital (IUH-IRB 12-0988).

Generation of JEV-NK PV

In this study, we analyzed the neutralization response to serum samples against the JEV Nakayama-NIH (NK) strain. Pseudotyped JEV containing the *env* gene of the JEV NK strain was generated using previously described methods (Lee *et al.*, 2009). Briefly, cells of the MuLV packaging cell line, TELCeB6 (Schnierle *et al.*, 1997), were transfected with pJEV-NK (E) using a calcium phosphate precipitation method. After an overnight incubation, the culture medium was replaced and cells were incubated for two additional days. The supernatants containing PVs were harvested by low-speed centrifugation (1,500×*g*, 5 min) to remove cell debris. Supernatant was aliquoted, stored at -80°C, and used as a virus stock.

Titration of JEV-NK PV

Virus titers were determined in Vero monolayer cells by infecting with 10 to 20 ml of culture supernatants and incubating for 36 h. All infections were done in triplicate. After incubation, the supernatant was removed and cells were fixed by incubating with 1% formaldehyde and 0.05% glutaraldehyde in phosphate-buffered saline (PBS, Invitrogen) for 10 min at room temperature. The cells were washed twice with PBS and incubated for at least 2 h at 37°C with freshly prepared staining solution consisting of PBS containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride, and 1 mg/ml of 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal, TakaRa, Japan). For routine analyses, X-Gal-stained blue cells were manually counted using an inverted microscope. Infectious units were determined by counting the number of blue cells (Kim *et al.*, 2001). Titer is expressed in infectious unit per milliliter (IFU/ml).

Neutralization assay

Vero cells (2×10^3 cells/well) were dispensed into 96-well plates and incubated for 16 h at 37°C in a humidified 5% CO_2 environment. Equal amounts of diluted serum sample and diluted JEV-PV were mixed and incubated at 37°C for 1 h. Thereafter, 100 ml of the neutralized liquid was inoculated into three wells per dilution and allowed to adsorb for 36 h at 37°C/% CO_2. X-gal staining was then performed as described above. Neutralizing titer was determined by testing serially diluted sera relative to concurrently tested control serum prepared from normal mice. The neutralization titer was expressed as the maximum serum dilution yielding a 50% reduction in virus.

Analysis method

The 50% plaque-reduction rate was computed using the equation,

$$K = 10^{\log Z-(50-Y)/(X-Y)}$$

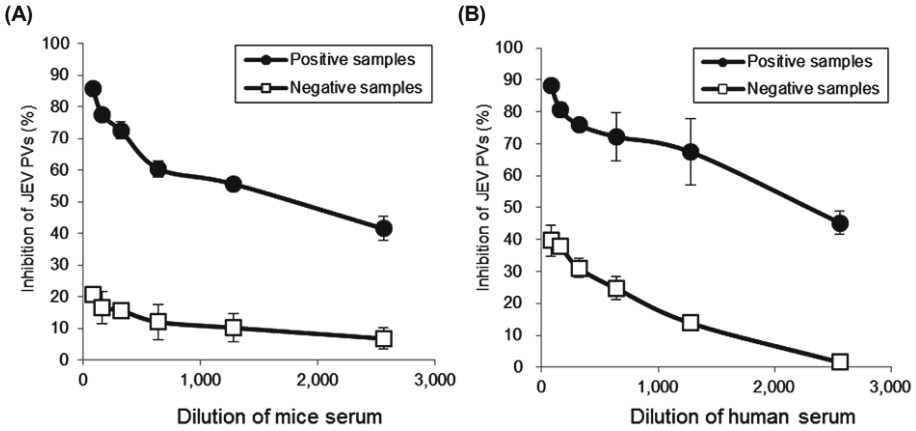

Fig. 1. Neutralizing activity of mouse and human serum against JEV-PV. Inhibition rates obtained by neutralization tests with mouse (A) or human (B) sera. Vero cells were inoculated with JEV-PV after pre-incubating with serially diluted mouse or human sera. The relative infectivity of JEV was calculated by measuring co-expressed β-galactosidase activity in supernatants after 36–48 h of inoculation. The results are shown as means ± SD of at least three independent assays.

established by the KCDC based on retrospective data, where K is the NAb titer, X is the reduction rate just above 50%, Y is the reduction rate just below 50%, and Z is the starting serum dilution. This equation uses the single point (one dilution) closest to the 50% plaque-reduction rate, based on the assumption that the relationship between plaque reduction and serum dilution is effectively a straight line showing the same degree of slope near this point.

Statistical analysis

SPSS (version 12.0) and STATA (version 9.0) software were used for statistical analyses. Parametric tests (Student's *t*-test, ANOVA, Pearson correlation) were used for comparisons and correlations among normally distributed variables. Mean or median values are presented with their respective 95% confidence intervals (CIs).

Results

Development of a PV neutralization assay against JEV in human sera

To establish a JEV neutralization assay using PVs, we produced JEV-PV encoding b-galactosidase and JEV-NK envelope (E) protein. In our previous study, we established a production system for JEV-PV using the JEV strain NK. JEV-PV was generated using the TELCeB6 packaging cell line, transfected with plasmid encoding the E protein of the JEV-NK strain. This PV also encodes a b-galactosidase gene, which allows detection of infected cells by staining with X-Gal. We optimized the titer of PV for the neutralization test and analyzed the specificity of neutralization using JEV-PV.

Cut-off value

To check the specificity of the JEV-PV assay, the neutralization curves were established with the mouse or human serum. The Naïve mouse serum was used as a negative control, and serum from JEV-NK-infected mice was used as a positive control. The positive serum inhibited JEV-PV in a concentration-dependent manner, whereas the negative serum did not inhibit JEV-PV at any dilution factor (Fig. 1A). Specificity was confirmed where the inhibition ratio of positive to negative mouse serum was greater than 4.2 to 6.1-fold. Using this method, we compared serum from a 1-yr-old child with serum sample 10 in Table 1. The ratio of mean inhibition values for human serum samples was 2.1- to 29.8-fold (Fig. 1B). Inhibition of JEV-PV was evaluated using serially diluted (1:10 to 1:2,560) serum samples with or without anti-JEV antibody. The inhibition by negative serum was less than 50% at a 1:10 dilution. This lack of inhibition by negative serum demonstrated that the PV assay is specific for anti-JEV antibodies. Serum sample 10 was subsequently used as a positive control because it was available in abundant supply for subsequent clinical testing. As a result of neutralization curves, the cut-off value of the PV assay was confirmed using sera with or without anti-JEV antibodies.

The limit of detection

To estimate the limit of detection, we diluted 30 human serum samples from 1:10 to 1:2,560. These samples could be

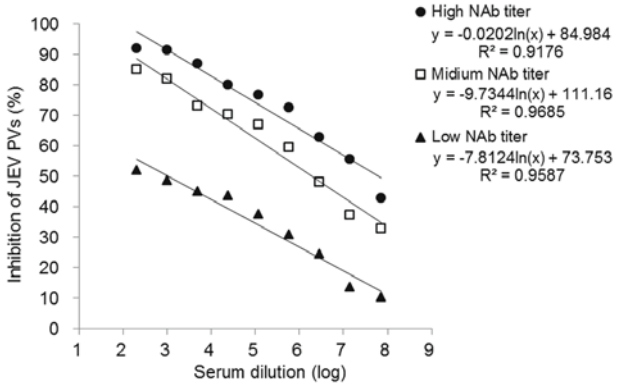

Fig. 2. Inhibition of diluted serum samples with a range of NAb titers. Serum samples with NAb titers in the high (640–2,560, n = 19), medium (80–640, n = 9), and low (10–80, n = 2) range were diluted from 1:10 to 1:2,500. The regression line was determined by the least-squares method.

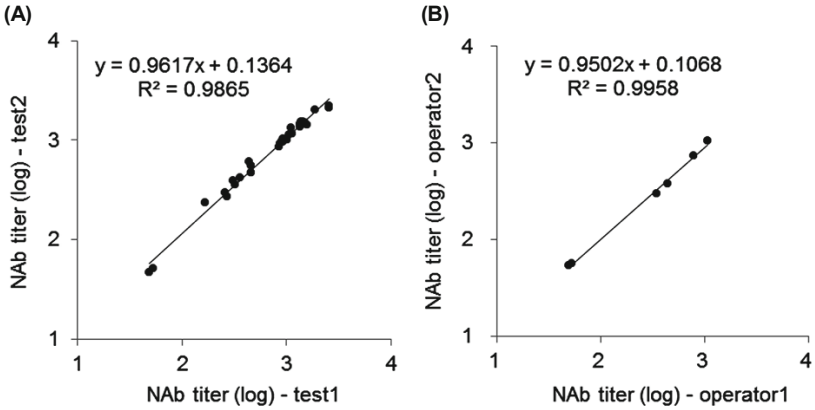

Fig. 3. Reproducibility of the PV assay. (A) A linear regression analysis showing the correlation of neutralization antibody titer obtained for 30 human serum samples tested on two different days by a single operator. (B) Correlation of neutralization antibody titer obtained for six human serum samples tested by two different operators.

grouped into those containing low-range (10–80), medium-range (80–640), and high-range (640–2,560) NAb titers. As shown in Fig. 2, correlation coefficients were high for all samples, with a suitable spread of data, independent of NAb titer and dilution. Specifically, linear regression analyses yielded values of R^2 = 0.9176 and coefficient = -0.020 (P = 0.0005) for high-range NAb titer (640–2,560) samples, R^2 = 0.9685 and coefficient = -9.7344 (P = 0.0005) for medium range NAb titer (80–640) samples, and R^2 = 0.9587 and coefficient = -7.8124 (P = 0.0005) for low-range NAb titer (10–80) samples. These results indicate a significant correlation between serial dilutions of serum and inhibition of JEV-PV in all cases. The limit of detection, defined as 50% inhibition of JEV-PV, was a 1: 2,560 dilution (samples in all NAb titer ranges yielded 0–43% of inhibition at a 1:2,560 dilution). Therefore, the dynamic range of this PV assay covers dilutions of 10 to 2,560.

Precision

Precision was investigated in terms of two considerations: repeatability and reproducibility. For repeatability, NAb titers were independently measured in 30 serum samples by a single operator in two separate experiments. As shown in Fig. 3A, a linear regression analysis showed a significant correlation (P < 0.0001) between the repeated measures (R^2 = 0.9865; 95% CI of slope = 0.9793–1.0722). For reproducibility, six serum samples were tested by two operators (Fig. 3B). A linear regression analysis demonstrated a highly significant correlation (P < 0.0001) between the NAb titers obtained by the two operators (R^2 = 0.9958; 95% CI of slope = 0.9622–1.1399). These results show that the PV-based neutralization assay is sufficiently precise and reproducible.

Correlation between the PRNT and PV assay

The PRNT and PV assay were compared by assessing 30 human serum samples with NAb titers ranging from 10 to 1,280 (Fig. 4). A regression analysis of all data (log-transformed values) showed a highly significant correlation (P < 0.0001), with R^2 = 0.915, 95% CI = 0.7722–1.2069, a slope of 0.67, and an intercept of 0.98. Consequently, both methods can be said to show a 1:1 correlation, an agreement that demonstrates the correlation between the PRNT and PV assay.

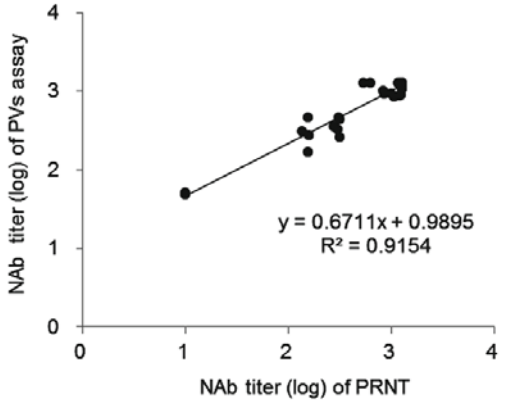

Fig. 4. Comparison of neutralizing antibody titers obtained using the PRNT and PV assay. Each test was conducted using 30 human serum samples. A linear regression analysis demonstrated a significant correlation (R^2 = 0.9154, P < 0.0001).

Discussion

The JEV NAb titer in the serum of vaccinated individuals is the parameter best correlated with protection from subsequent viral infection. However, the PRNT method suffers from limited sensitivity and non-specificity; in addition to posing a biohazard, it is also technically demanding and labor-intensive, resulting in variable results (Martin *et al.*, 2008).

Conventional PRNT assay depend on the level of virus replication, risk of infection by live virus, and different assay conditions (e.g., agar overlay onto the cell monolayer, plaque visualization, and counting plaques). Additionally, in the PRNT assay requires a long time (about 7 days). Current guidelines suggest a procedure for laboratories interested in using the PRNT, but the lack of a standard PRNT poses a hurdle for comparing results between laboratories and defining a threshold value to use as a true serological correlate of protection. Importantly, however, virus-neutralizing antibody titer, as determined by the PRNT, is considered

the best immune correlate of protection for flaviviral infections (Roehrig *et al.*, 2008).

In order to safely, conveniently, and shorten the assay period, we established JEV-PV neutralization assay. We have confirmed the JEV-PV neutralization assay with human serum samples by comparison with the PRNT. Compared with the PRNT, the PV neutralization assay required less than half the time (2 days) needed to test for neutralizing antibodies. Our JEV-PV neutralization assay provides a convenient way for detecting JEV infected cells as X-gal staining and observation. Additionally, this JEV-PV neutralization assay is carried out in 96-well plates, providing a more cost-effective and less labor-intensive platform for investigating large numbers of samples.

JEV-NAb titer ranges for positive and negative control sera were established by evaluating 30 human sera using the PV assay. Human sera from two infants without JEV vaccination were also evaluated as negative controls. JEV-specific NAbs were detectable in these two negative control sera using the PV assay. The mean of NAb titers for these negative sera was 49.4±0.15; this compares with a $PRNT_{50}$ NAb titer of 10 considered by WHO vaccine guidelines to be protective (Hombach *et al.*, 2005). Inter-operator reproducibility of NAb titers was determined from repeated-measures experiments. For repeatability studies, 28 aliquots of human serum and two aliquots of a negative control serum were tested separately in the PV assay. Tests of the repeatability of the negative serum pool yielded a coefficient of variation (CV) of 10.3. These same negative serum pools were analyzed on several different days to estimate reproducibility. We deduction; thus, a NAb titer of greater than 50 in the PV assay is considered to be positive. To become a standard and widely-used neutralization assay, enabling even large numbers of JEV-negative serum samples to be tested and evaluated.

As reported here, the performance of the PV assay is comparable to that of the PRNT, as evidenced by the high correlation between titers determined by both assays. Importantly, it is more efficient and shows greater the limit of detection, repeatability, and reproducibility than does the PRNT.

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