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Comparison of plaque reduction and focus reduction neutralization tests for the measurement of neutralizing antibody titers against japanese encephalitis virus

Younhee Park [a], Ah-Ra Kim [b], Yun-Ho Hwang [c], Hyejung Yang [a], June-Woo Lee [b], Mi Young Kim [d], Hwa Su Kim [e], Gyung Tae Chung [f], Jung Sik Yoo [g], You-Jin Kim [c], Dokeun Kim [c], Hyeran Won [c,*]

[a] Department of Laboratory Medicine, Yonsei University College of Medicine, Seoul, Republic of Korea
[b] Division of Vaccine Clinical Research, National Institute of Health, Korea Centers for Disease Control and Prevention Agency, CheongJu, Chungcheongbuk-do, Republic of Korea
[c] Division of Infectious Diseases Vaccine Research, National Institute of Health, Korea Centers for Disease Control and Prevention Agency, CheongJu, Chungcheongbuk-do, Republic of Korea
[d] Division of Vaccine Development Coordination, National Institute of Health, Korea Centers for Disease Control and Prevention Agency, CheongJu, Chungcheongbuk-do, Republic of Korea
[e] Division of HIV/AIDS prevention and Control, Korea Diseases Control and Prevention Agency, CheongJu, Chungcheongbuk-do, Republic of Korea
[f] Center for Infectious Disease, National Institute of Health, Korea Centers for Disease Control and Prevention Agency, CheongJu, Chungcheongbuk-do, Republic of Korea
[g] Division of Antimicrobial Resistance, National Institute of Health, Korea Centers for Disease Control and Prevention Agency, CheongJu, Chungcheongbuk-do, Republic of Korea

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ABSTRACT

Japanese encephalitis is prevalent throughout the temperate and tropical regions of Asia and is caused by the Japanese encephalitis virus (JEV), a mosquito-borne viral pathogen. The plaque reduction neutralization test (PRNT) is currently recommended as the gold standard test for detecting human antibodies against JEV. The plaque assay is the most widely used method for detecting infectious virions and involves counting discrete plaques in cells. However, it is time-consuming, and results can be subjective (owing to analyst variability during manual plaque counting). The focus reduction neutralization test (FRNT), which is based on an immunocolorimetric assay, can be used to automatically count foci formed by the JEV. Here, we compared the efficacy of PRNT and FRNT in measuring the neutralizing antibody titers using 102 serum samples from vaccinated and unvaccinated individuals. We observed positive correlations between these neutralization assays against the Nakayama and Beijing strains (R^2 = 0.98 and 0.77, respectively). Thus, FRNT may be preferable to PRNT for evaluating the efficacy of JEV vaccines in large-scale serological studies.

1. Introduction

Japanese encephalitis (JE) is caused by the Japanese encephalitis virus (JEV), a member of the genus *Flavivirus* within the family *Flaviviridae*. JEV is a single-stranded, positive-sense RNA virus with an 11 kb genome that contains a single long open reading frame that encodes three structural (C, prM, and E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins (Turtle and Solomon, 2018). Four types of JE vaccines are currently approved for human use: a mouse brain-derived inactivated vaccine (Nakayama), a Vero cell culture-derived inactivated vaccine (Beijing) [GC Biopharm Corporation: Japanese encephalitis vaccine and BORYUNG: Japanese encephalitis vaccine], a cell culture-derived live attenuated vaccine (SA 14–14–2) [GLOVAX: CD.JEVAX], and a live attenuated recombinant vaccine (SA 14–14–2) [Sanofi: IMOJEV]. The Nakayama vaccine was introduced by Korea's National Immunization Program (NIP) in 1985, the SA 14–14–2 vaccine in 2014, and the Beijing vaccine in 2015 (KDCA, 2020; Lee et al., 2016). The SA 14–14–2 is not currently included in the NIP (KDCA, 2020).

The plaque reduction neutralization test (PRNT) is the best and the

* Correspondence to: 212 Osongsaengmyeong 2-ro, Osong-eup, Heungdeok-gu, Cheongju-si, Chungcheongbuk-do 28160, South Korea.
E-mail address: hw403@korea.kr (H. Won).

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most widely accepted method for measuring virus-neutralizing and protective antibodies against JEV (Hills et al., 2009; Shyu et al., 1997]. However, PRNT is technically demanding and is limited by the large number of serum samples to be processed, which is a prerequisite in seroepidemiological studies. There have been many attempts to develop alternative experimental methods to PRNT. In particular, the focus reduction neutralization test (FRNT) based on an immuno-colorimetric assay (ICA) has been introduced for diverse viruses (Bannai et al., 2013; Canakoglu et al., 2013; Katzelnick et al., 2018; Vaidya et al., 2010). FRNT can be used to visualize viral foci via immune staining before plaque formation, resulting in a shorter test period than PRNT.

Here, we evaluated the suitability of FRNT as a standard assay for the detection of JEV-neutralizing antibodies by exploring the relationship between FRNT and PRNT results regarding the standardized plaque assay for JEV.

2. Materials and methods

2.1. Reagents

The following reagents were used in this study:

1. Vero E6 cells (ATCC, catalog # CRL-1586)
2. Dulbecco's modified Eagle medium (DMEM; Gibco, catalog # GIB-11995–073)
3. Minimum Essential Medium (MEM; GenDEPOT, catalog # CM041-050)
4. MEM (Temin's modification; 2X; Gibco, catalog # 11935046)
5. Penicillin-Streptomycin (P/S), Liquid (10,000 U·mL^{-1}; Gibco, catalog # GIB-15140–122)
6. Fetal bovine serum (FBS) USA, Certified, Heat-Inactivated (Gibco, catalog # GIB-10082–147)
7. Agarose (Lonza, catalog # 50004)
8. Carboxymethylcellulose (CMC; Sigma, catalog # C5678)
9. Phosphate-buffered saline (PBS; GenDEPOT catalog # P2101-100)
10. 20X PBS with Tween 20 (Thermo Fisher catalog # 28352)
11. Paraformaldehyde Solution 4% (BIOSESANG, catalog # P2031)
12. Methanol (Merch, catalog #: 1.06009.1011)
13. Blocker bovine serum albumin (BSA) in PBS (10X; Thermo Fisher, catalog # 37525)
14. Viral JEV NS1 Antibody (R & D systems, catalog # MAB10006)
15. Goat anti-Mouse immunoglobulin (IgG; H+L) Secondary Antibody, horseradish peroxidase (HRP) conjugate (Invitrogen, catalog # 62–6520)
16. Crystal violet solution (Sigma, catalog # V5265)
17. TrueBlue Peroxidase substrate (Sera Care, catalog # 5510–0030)
18. JE Detect IgG enzyme-linked immunosorbent assay (ELISA; InBios, catalog # JEGS-1)

2.2. Clinical specimens and ethics statement

Negative control serum samples were collected from unvaccinated individuals (n = 39, 12 adults and 27 infants aged <1 year). Test samples also included 53 serum samples from healthy blood donors aged 2–7 years who had been received at least one dose of JE vaccine.

This study was conducted in strict adherence to the Korean Centers for Disease Control and Prevention Agency (KDCA) guidelines for experiments using human sera, and the study protocol was approved by the institutional review boards of the KDCA (approval number: 2020–06–01-PE-A) and the Severance Hospital (Seoul, Korea) (approval number: 4–2019–1225).

The serum samples were collected by age according to the JEV schedule and residuals from general medical checkups. We also collected the vaccination histories of specimens using deidentified information according to the ethical policy.

2.3. Cell culture and virus harvest

Vero E6 cells were obtained from ATCC (CRL-1586). They were cultured at 37 °C under 5% carbon dioxide (CO_2) in DMEM supplemented with 10% (v/v) FBS and 1% (w/v) P/S. Vero E6 cells were inoculated with the Nakayama and Beijing JEV vaccine strains in MEM supplemented with 2% (v/v) FBS and 1% (w/v) P/S. The infected cells were harvested when the virus-specific cytopathic effect was 80%, through two rounds of freeze-thawing. The JEV-containing media were stored at − 80 °C until use.

2.4. Virus titration

2.4.1. Plaque assay

Vero E6 cells were seeded at a density of 2×10^5 cells per well in 12-well plates containing DMEM supplemented with 10% FBS and 1X P/S. They were incubated at 37 °C in 5% CO_2 for 24 h. The serially diluted JEV stock virus was inoculated into Vero E6 cells for 2 h in MEM at 37 °C in 5% CO_2. The inoculum was discarded, and the cell monolayer was overlaid with 0.8% agarose in MEM containing 2% FBS and 1% P/S. The cells were cultured at 37 °C in 5% CO_2 for 72 h, and plaque appearance was monitored. Plaques became visible three days later, following which cells were fixed with 4% formaldehyde in PBS and stained permanently with 1% crystal violet solution. Viral titers were calculated in plaque forming units (PFUs)·mL^{-1}.

2.4.2. Focus forming assay

Vero E6 cells were seeded at a density of 2×10^4 cells per well in 96-well plates containing DMEM supplemented with 10% FBS and 1X P/S. They were then kept at 37 °C in 5% CO_2 for 24 h. The serially-diluted JEV stock virus was inoculated into Vero E6 cells for 2 h, in MEM at 37 °C in 5% CO_2. The inoculum was then removed, following which the cell monolayer was overlaid with 1.5% CMC media in MEM containing 2% FBS and 1% P/S. The cells were then maintained at 37 °C, in 5% CO_2, for 48 h. Next, the cells were fixed with 100% methanol at 4 °C for 30 min, before being washed with ice-cold PBS and blocked with 100 μL·well^{-1} blocking buffer (2.5% BSA, 0.5% FBS and 0.1% Tween-20 in PBS) for 30 min at room temperature (RT). They were then incubated with 50 μL·well^{-1} of JE virus NS1 antibody, which was diluted 1:1000 in blocking buffer, for 2 h at RT. The plate was then washed three times with PBS plus 0.1% tween-20 (PBST), before being incubated with 50 μL·well^{-1} of goat anti-mouse IgG HRP-labeled antibody (diluted 1:2000 in PBS), for 1 h, at RT. The plate was washed three times with PBST, following which 50 μL of TrueBlue peroxidase substrate was added for 30 min, at RT. The substrate solution was then aspirated. The blue viral foci were automatically counted using an ImmunoSpot® S6 universal analyzer with ImmunoSpot® software v.7. Viral titers were calculated in terms of the focus forming units·mL^{-1}.

2.5. ELISA

JEV-specific IgG was determined using InBios JE Detect IgG ELISA (Seattle, WA, USA) according to the manufacturer's protocol. Both positive and negative antigens were included to validate the test and control for background nonspecific reactivity with each antigen. The results were interpreted based on the Japanese Encephalitis Recombinant Antigen (JERA)/Normal Cell Antigen (NCA) ratio as follows: negative (<2), equivocal (2−5), or positive (>5).

2.6. PRNT

Neutralizing assays were performed with serum heat-inactivation at 56 °C for 30 min. Sera were diluted two-fold from their initial dilution of 1:20 in 96-well plates. The serially diluted sera were incubated with 40–60 PFUs of the Nakayama or Beijing strain for 2 h at 37 °C under 5% CO_2. The mixtures were then absorbed into Vero E6 cells cultured in 12-

well plates for 2 h. The average numbers of spots were calculated from duplicate experiments, with neutralizing antibody titers defined as the dilution factor corresponding to 50% plaque reduction compared with the control.

The 50% plaque reduction titer (PDD50) for each individual serum sample was calculated as follows: PDD50 = DL + [P_{50} - PL)(DH - DL)/(PH - PL)], where DL is the reciprocal of the lower dilution bracketing the 50% endpoint; PL is the number of plaques at the lower dilution bracketing the 50% endpoint; DH is the reciprocal of the higher dilution bracketing the 50% endpoint; PH is the number of plaques at the higher dilution bracketing the 50% endpoint; and P50 is the number of plaques at the 50% endpoint (Perng et al., 1999).

2.7. FRNT

Neutralizing assays were performed with serum heat-inactivation at 56 °C for 30 min. Sera were diluted two-fold from the initial dilution of 1:20 in 96-well plates. The serially diluted sera were incubated with 40–60 foci of the Nakayama or Beijing strain for 2 h at 37 °C under 5% CO_2. The mixtures were then absorbed into Vero E6 cells cultured in 96-well plates for 2 h. The average numbers of foci were calculated from triplicate experiments, with neutralizing antibody titers defined as the dilution factor corresponding to 50% plaque reduction compared with the control. The 50% plaque reduction titer was calculated using the same formula used for PRNT (see Section 2.6).

2.8. Statistical analysis

Statistical analyses were performed using Graph Pad Prism v. 6.0. The correlations between two assays were computed via linear regression using Spearman's correlation coefficient. Statistical significance was determined using the two-tailed test and was set at $P < 0.05$.

3. Results

3.1. JE vaccination history

The JE vaccination history was investigated to confirm whether the positive conversion rate of neutralizing antibodies against JEV was due to JE vaccination. The samples were obtained from both unvaccinated and vaccinated individuals; detailed information on the specimens is shown in Table 1 and Supplementary Table S1.

3.2. JE IgG

Each specimen was measured for JE-specific IgG to confirm vaccine-induced immunity. In the unvaccinated group, two samples were equivocal, and all the other samples were negative. In the vaccinated group, 21 samples were negative, 25 were equivocal, and seven were positive according to the results of JE Detect IgG analysis (Fig. 1). When analyzed using JE Detect IgG ELISA, the unvaccinated group either had negative or equivocal results. In contrast, the vaccinated group showed mixed results as negative, equivocal, and positive. These results show

Table 1
JE vaccination history of specimens ($N = 92$)

Group	Number of specimens		Types of JE vaccine (Vaccination numbers)
Unvaccinated	39		–
Vaccinated	53	1	SA 14–14–2 (1)
		10	SA 14–14–2 (2)
		1	SA 14–14–2 (1) Beijing (3)
		10	Beijing (2)
		19	Beijing (3)
		1	Nakayama (2) Beijing (1)
		11	Nakayama (3) Beijing (1)

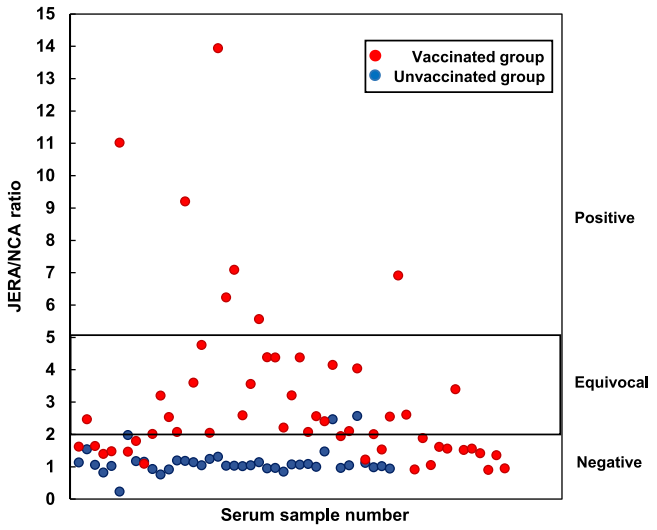

Fig. 1. Diagnosis of Japanese encephalitis virus (JEV)-specific IgG from 92 human serum samples collected from 39 unvaccinated individuals and 53 vaccinated individuals. The IgG enzyme-linked immunosorbent assay (ELISA) output is expressed as the JERA/NCA ratio and classified as negative (<2), equivocal (2–5), or positive (>5).

that the JE IgG response did not increase following vaccination, regardless of the type of JE vaccine.

3.3. Comparison between JE IgG and PRNT and between JE IgG and FRNT

We next measured the responses of neutralizing antibodies against the Nakayama or Beijing JE vaccines using the same panels of samples measured for JE IgG. We did this to assess the relationships between the neutralizing antibodies detected by PRNT or FRNT and JE IgG (as determined by the JE Detect IgG ELISA). Regarding the Nakayama JE vaccine, the Pearson correlations between JE IgG and PRNT and between JE IgG and FRNT were weak (0.35 and 0.38, respectively). For the Beijing JE vaccine, the Pearson correlations between JE IgG and PRNT and between JE IgG and FRNT were moderate (0.55 and 0.56, respectively). We excluded the samples with undetected results (PDD50 <20) via PRNT or FRNT (Fig. 2). Thus, eight of the total 39 samples in the unvaccinated group and 46 of the total 53 samples in the vaccinated group were analyzed.

3.4. Comparison between PRNT and FRNT

The neutralizing antibody titers between the two assays were not significant (Fig. 3). One consistent trend was that the neutralizing antibody titer measured using PRNT was lower than that measured by FRNT. Furthermore, strong correlations were observed between the two assays against the Nakayama and Beijing strains ($R = 0.98$ and 0.77, respectively). These results indicate that FRNT represents a reliable approach for evaluating JE vaccines. Results from undetected samples (PDD50 <20) were excluded from the analysis used in the above-mentioned PRNT; the number of analyzed samples was the same as that mentioned in Section 3.3.

4. Discussion

PRNT is currently widely used as the gold standard assay for measuring neutralizing antibody titers against flaviviruses. However, it is relatively time-consuming and labor-intensive and has a low throughput. Moreover, many of the variables of PRNT, such as virus passage, cell lines, and the use of a complement, can affect the assay

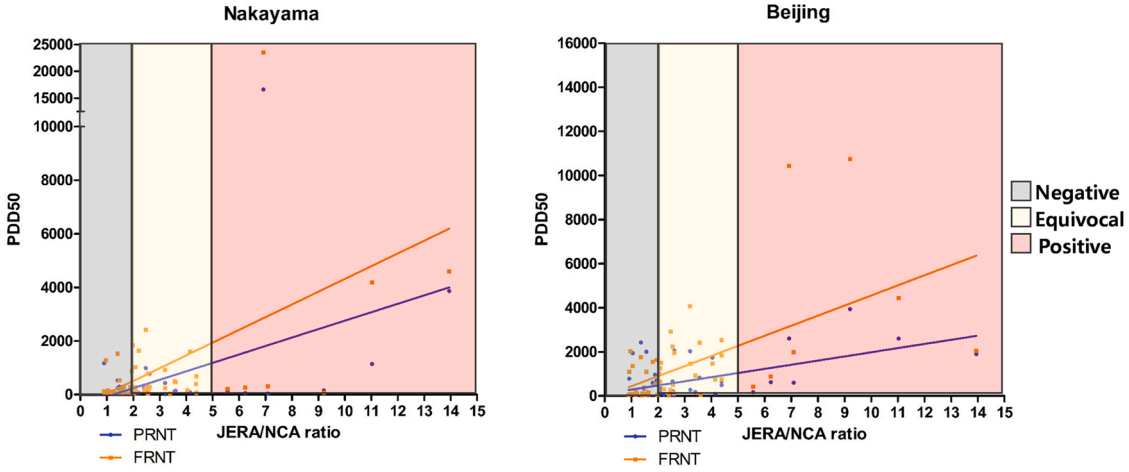

Fig. 2. Comparisons between JE IgG ELISA and plaque reduction neutralization test (PRNT) and between JE IgG ELISA and focus reduction neutralization test (FRNT). Correlations between JE-specific IgG ELISA and PRNT and between JE IgG ELISA and FRNT against JEV were analyzed using 54 human serum samples. The blue solid line indicates a complete correlation between JE-specific IgG ELISA and PRNT. The 95% confidence interval is 0.09405–0.5672, and the *p* value is 0.0088 between JE-specific IgG ELISA and PRNT. The 95% confidence interval is 0.1197–0.5845, and the *p* value is 0.0051 between JE-specific IgG ELISA and FRNT against Nakayama. The orange line indicates a complete correlation between JE-specific IgG ELISA and FRNT. The 95% confidence interval is 0.3296–0.7121, and the *p* value is < 0.0001 between JE-specific IgG ELISA and PRNT. The 95% confidence interval is 0.3467–0.7215, and the *p* value is < 0.0001 between JE-specific IgG ELISA and FRNT against Beijing. Statistical significance was calculated using two-tailed test.

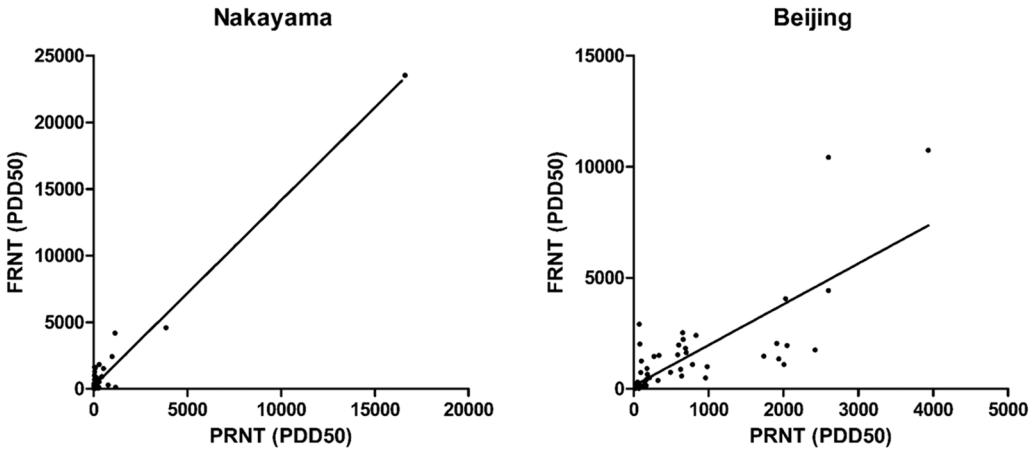

Fig. 3. Comparison of correlations between PRNT and FRNT analyzed using 54 human serum samples against the Nakayama and Beijing JEV strains. The solid line indicates complete correlations between PRNT and FRNT. The 95% confidence interval is 0.9684–0.9893, and the *p* value is < 0.0001 between PRNT and FRNT against Nakayama. The 95% confidence interval is 0.6370–0.8623, and the *p* value is < 0.0001 between PRNT and FRNT against Beijing. Statistical significance was calculated using two-tailed test.

results. To overcome these deficiencies, improved tests that assess the neutralization of diverse flaviviruses are being developed, such as a FRNT, which is based on an ICA (Jirakanjanakit et al., 1997; Smith and Hirsch, 2020; Tun et al., 2016). However, any new assay for measuring JEV-neutralizing antibody titers needs to be validated for consistency against PRNT. Although FRNT and PRNT are based on the same principle, they use different volumes of reagents and serum and their counting method after the visualization steps is also different. These minor differences make FRNT more advantageous than PRNT. As FRNT is performed in a 96-well plate, it allows the analysis of larger numbers of samples in a single batch than that by PRNT while utilizing fewer samples and reagents and taking up less space in incubators.

In this study, we used FRNT to measure neutralizing antibody titers against JEV and found that FRNT obtained comparable results to PRNT. We tested the correlations between PRNT and FRNT against two JE vaccine strains, Nakayama and Beijing, while taking into consideration the vaccination history of the participants. The specimens were collected from unvaccinated individuals (n = 39, 12 adults and 27 infants aged <1 year) to serve as negative controls and from healthy blood donors (n = 53) aged 2–7 years who had received at least one dose of JE vaccine as positive controls. Some negative samples from infants aged < 1 year were confirmed to have neutralizing antibodies, which could have been derived from their mothers.

We found that there was a significant agreement between the two methods against both Nakayama and Beijing strains ($R = 0.98$ and 0.77, respectively). The quantitative JE IgG units obtained via ELISA (Table 1) did not correlate well with the 50% neutralization titers obtained by either PRNT (R =0.35 and 0.55) or FRNT (R = 0.38 and 0.56) against the Nakayama and Beijing strains, respectively. A PRNT50 threshold of $\geq$ 1:10 is accepted as an indication of seroprotection (Feroldi et al., 2012; World Health Organization, 2015).

This study has some limitations. First, we analyzed residual sera samples after routine medical examinations; thus, the serum volumes were often insufficient. Second, FRNT exhibited a higher sensitivity than PRNT regarding JEV detection. Therefore, we performed PRNT and FRNT at two-fold dilution, starting with a dilution of 1:20. Thus, a cut-off verification test is needed in the future, and studies with larger sample sizes and more detailed vaccination information are required to confirm our findings.

Overall, this study demonstrated that FRNT could replace the PRNT assay as the gold standard for measuring neutralizing antibody titers. FRNT could also be used to monitor the temporal waning of protective

neutralizing titers for large-scale seroepidemiological studies of vaccinated individuals. Furthermore, FRNT could aid the evaluation of the effectiveness of JE vaccines, thereby helping the development of vaccination strategies, such as reducing the number of required vaccinations.

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CRediT authorship contribution statement

Younhee Park: Conceptualization, Formal analysis, Investigation, Writing – review & editing. **Ah-Ra Kim:** Methodology, Investigation. **Yun-Ho Hwang:** Methodology, Investigation. **Hyejung Yang:** Methodology, Investigation. **June-Woo Lee:** Conceptualization, Investigation. **Mi Young Kim:** Conceptualization, Investigation. **Hwa Su Kim:** Conceptualization, Investigation. **Gyung Tae Chung:** Conceptualization, Investigation. **Jung Sik Yoo:** Conceptualization, Investigation. **You-Jin Kim:** Conceptualization, Investigation. **Gyung Tae Chung:** Conceptualization, Investigation. **Jung Sik Yoo:** Conceptualization, Investigation. **You-Jin Kim:** Conceptualization, Investigation. **Dokeun Kim:** Conceptualization, Investigation. **Hyeran Won:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing, Resources, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2022.114540.

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