Identification of SARS-CoV-2 Neutralizing Potency of Human Convalescent Plasma Using Pseudovirus Neutralization Assay

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Abstract

Convalescent plasma samples that can be collected from individuals after the resolution of infection and vaccination are an invaluable source of neutralization antibodies against the virus. Although plaque reduction assay with replicative virus is the gold standard of analyzing neutralization potency of convalescent plasma, it is a technically demanding procedure requiring high biosafety level (BSL-3/4) laboratory and equipment. The abundance of neutralizing antibodies varies among individuals, therefore fast and reliable methods to identify neutralization potency of plasma samples are needed. In this paper, G-protein deficient vesicular stomatitis virus (VSV-ΔG) carrying a C-terminal 21 amino acid truncated severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Spike protein was generated for pseudovirus neutralization assay. We analyzed SARS-CoV-2 neutralizing potency of vaccinated human convalescent plasma samples (n=13) and plasma samples of healthy people (n=2). Human convalescent plasma samples were examined against the ancestral Wuhan strain and two SARS-CoV-2 variants (B.1.1.7, and B.1.351) using a VSV-ΔG-Sdel21 pseudovirus and Vero E6 cell line. Neutralization values against pseudotyped virus were compared to those of plaque reduction assay against authentic virus. The serum neutralizing titer of convalescent plasma measured by pseudovirus assay has a good correlation with that measured by plaque reduction assay ($R^2 = 0.7$). The pseudovirus assay is safer and timesaving than the replicative virus-based plaque reduction assay, and has several advantages in evaluating a new vaccine, newly emergent variants, and approved vaccine efficacy against variants of concern as well as in viral fusion-focused treatment analysis that can be performed under BSL-2 conditions.

Introduction

Serum neutralization corresponds protection against viral infection after vaccination or natural infection. However, high levels of mutation observed in viral glycoprotein of different variants could prevent effective protection provided by neutralization serum. Therefore, immediate determination of serum neutralization potency against different variants is crucial (Wu et al., 2020). Particularly, new variants have begun to emerge for disease caused by global infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) since 2019. It has been reported that mutations on the major neutralization target of these variants alter serum-neutralizing activity induced by early strains or vaccines (Kuzmina et al., 2021; Robbiani et al., 2020). Some of these mutations can provide an escape from immune response by decreasing monoclonal antibody (mAb) neutralization (Liu et al., 2021).

Due to the high risk of SARS-CoV-2 infection, cultivation of authentic viruses requires laboratories with at least level 3 biological safety (BSL3) equipped with negative pressure systems. This limitation prevents

to perform neutralization assays in many research centers, which only has BSL2 laboratories. Pseudotyped virus neutralization assay is an alternative method based on packaging a reliable replication-defective pseudovirus with Spike protein to mimic the entry of the authentic virus. Thereby, pseudoviruses can be contained in BSL2 conditions to perform neutralization assays (Chen and Zhang, 2021; Li et al., 2018). In the broad extent, pseudoviruses can be utilized in seroepidemiological studies, vaccine and monoclonal antibody development, screening of viral entry inhibitors, and fundamental virological methods (Ou et al., 2020). Several convenient pseudotyped virus for SARS-CoV-2 have been reported, such as human immunodeficiency virus (HIV)-based lentiviral particles (Donofrio et al., 2021), murine leukemia virus (MLV)-based retroviral particles (Chen and Zhang, 2021), or vesicular stomatitis virus (VSV)-based systems (Nie et al., 2020; Salazar-García et al., 2022) with higher performance for evaluating the efficacy of therapeutic drugs and vaccines (Salazar-García et al., 2022). The results of such pseudovirus neutralization tests are very closely correlated to those of authentic virus measurements. Also, tracking the changes in Spike glycoprotein using pseudovirus neutralizing assays is relatively easy (Schmidt et al., 2020).

From the beginning of the pandemic, DNA sequencing data of virus glycoproteins is utilized to identify individual mutations in SARS-CoV-2 (Schrörs et al., 2021). One of the first variants called B.1.1.7 was first emerged in the United Kingdom and had multiple mutations in the target regions of neutralizing antibodies such as receptor binding domain and N-terminal domain of Spike. SubsequentlyB.1.351 variant has been identified in South Africa with additional mutations. B.1.1.7 and B.1.351 variants share key mutations in the RBD (N501Y, D614G), but B.1.351 has additional changes causing widespread escape from mAbs (E484K, K417N) (Harvey et al., 2021; Zhou et al., 2021).

In this paper, we evaluated human convalescent plasma with different serum neutralizing activities using pseudotyped VSV-ΔG virus carrying Spike variants (*Wuhan* strain, B.1.1.7, and B.1.351). First, we made a series of point mutations in Spike sequence of SARS-CoV-2 to obtain global variants. Second, we generated pseudotyped viruses usingSpike of ancestoral Wuhan strain and two variants and utilized to evaluate neutralization activity of human serum samples. Neutralization assay using Spike pseudotyped VSV-ΔG virus was found to correlate with plaque assay using SARS-CoV-2 authentic virus. Additionally, Spike pseudotyped VSV-ΔG virus was sufficient to discriminate serum responses against different variants of the virus.

Materials and Methods

Plasmid construction

The expression vector for SARS-CoV-2 Spike (pTWIST-EF1-alpha-SARS-CoV-2-S-2xStrep vector) was kindly provided by Dr. Nevan Krogan. Spike gene was codon optimized to improve the expression efficiency in mammalian cells (Genbank: MN985325). A pMD2.G lentiviral plasmid containing Spike gene was generated. Briefly, forward primer 5'-ATAGAATTCGCCGCCACCATG-3' and reverse primer 5'-ATAGAATTCTCATCAACTACCGCAAGAACAACAACC-3' were used to amplify mutant Spike protein with 21 amino acid deletion in C-terminal. To clone Spike gene successfully, TA cloning was applied. In the first step, PCR reaction was set up to add adenine bases to 3'OH terminal of Spike gene. The amplified product was ligated into pGEM-T easy vector system. In the second step, Spike gene was restricted by *EcoRI* and ligated into pMD2.G vector. Spike gene with C-terminal 21 amino acid deletion (pmD2.SpikeCdel21) was confirmed with Sanger DNA sequencing.

B.1.1.7 (*Alpha*) variant including ΔH69-V70, N501Y, D614G, ΔY144 mutations and B.1.351 (*Beta*) variant including E484K, N501Y, D614G, K417N mutations of SARS-CoV-2 were constructed in pmD2.SpikeCdel21 plasmid by using site-directed mutagenesis. PCR conditions were set up by following procedures of Phusion High-Fidelity Polymerase (Thermo Sci., F530L) and Quick-change Site-directed Mutagenesis Kit (Startagene, Agilent, 200518). Next, the template was digested with *DpnI* to eliminate parental DNA. The constructed vector was transferred into *E. coli XL-1 Blue* competent cells. Following the picking up colonies, plasmids were sequenced, and mutations were confirmed. Sequences of mutation primers and sequences of plasmid constructs are given in **Supporting Information File.**

Cell culture

HEK (Human Embryonic Kidney) 293T cells (ATCC, CRL-321) and Vero E6 (Monkey African Green Kidney) (ATCC, CRL-1586) cells were cultured in Dulbecco's modified Eagle medium (DMEM, PAN Biotech, P04-3500) supplemented with 10% fetal bovine serum (PAN Biotech, FBS standard, P30-3306) and %1 L-glutamine (200 mM; PAN Biotech, P04-80100) and 1% penicillin/streptomycin (Pan Biotech, P06-07100) at 5% (v/v) CO_2, 37 °C incubator.

Plasma samples

Human convalescent plasma samples were obtained from volunteer donors after 2 months post-vaccination **(Table 1)** . Plasma samples were heat-inactivated at 56°C for 30 minutes before using for neutralization assays. Sinovac and BioNTech-Pfizer mRNA vaccine BNT162b2 were used for vaccination in Turkey.

Table 1. Vaccination status of human plasma samples used for neutralization assays

Plasma samples	Vaccination
1	Sinovac, 2 dose
2	Sinovac, 2 dose
3	Sinovac, 2 dose
4	Sinovac, 2 dose; BioNTech, 1 dose
5	Sinovac, 2 dose; BioNTech, 1 dose
6	Sinovac, 2 dose; BioNTech, 1 dose
7	Sinovac, 2 dose
8	Sinovac, 2 dose; BioNTech, 1 dose
9	BioNTech, 2 dose
10	BioNTech, 2 dose
11	BioNTech, 2 dose
12	Sinovac, 2 dose
13	Sinovac, 2 dose
14	Healthy control
15	Healthy control

Plaque reduction neutralization assay

Serum samples (non-denatured) were diluted in DMEM from 1:10 to 1:320. SARS-CoV-2 virus was diluted in DMEM to a final concentration of 10^{-3} pfu/ml. 300 µl serum and 300 µl diluted virus were mixed and incubated in37 °C incubator at 5% (v/v) CO_2 for 1 hr. Then, the serum-virus mixture was added to Vero E6 cells and incubated in, 37 °C incubator at 5% (v/v) CO_2 for 1 hr. Serum-virus mixture was discarded from the plate and the plate was covered with 2% methylcellulose and DMEM at 1:2 ratio and incubated for 4 days. Methylcellulose was discarded and cells are washed with 1xPBS. Then, 4% PFA was used for cell fixation at room temperature for 20 minutes. After fixation, cells were stained with crystal violet solution for 30 minutes at room temperature. Plaques were counted after washing with PBS to remove the excessive stain. The number of plaques were used to calculate virus neutralization titers.

Generation of SARS-CoV-2 pseudotyped variants

rVSV-ΔG-G* (G*: VSV glycoprotein) and VSV-ΔG-Spike (carrying Spike protein of coronavirus instead of VSV glycoprotein) virus were produced according to the protocol provided by Whitt in 2010 (Whitt, 2010). BHK-21/WI-2 cells (Baby Hamster Kidney-21/ Clone WI-2, Kerafast, EH1011) were infected with vaccinia virus (ATCC, strain vTF7-3, VR-2153^{TM}) for 1 h, followed by co-transfection of five plasmids: VSV-ΔG, together with VSV accessory plasmids encoding for VSV-N, P, L, and G proteins (Kerafast), all of which were under T7 promoter control. The primary transfection was performed using Lipofectamine 2000. BHK-21/WI-2 cells were transfected with Spike plasmid to assist in creating passage 1 (P1). Forty-eight hours following primary transfection, supernatant containing the recovered VSV-Spike was collected, centrifuged at

1300 × g × 5 min to remove cell debris. Total supernatant was collected, centrifuged, and used for sequential passaging in Vero E6 cells to eliminate parental virus (Yahalom-Ronen et al., 2020), as shown in **Figure 1** . VSV-ΔG-Spike was propagated in DMEM containing 5% FBS (reduced serum media), MEM NEAA, 2mM L-glutamine, and 1% P/S.

SARS-CoV-2 pseudovirus infection for viral entry and titration

To determine viral titer, Vero E6 cells were seeded in a 24-well-plate before the day of infection. 100 µl VSV-ΔG-Spike pseudotyped virus was diluted 3-fold serial dilutions up to 1:243 using DMEM 5% FBS as diluent. After 24 hours, GFP positive cells were counted in the last wells to calculate the TU/ml.

For viral entry assay, medium was replaced with 200 µl cell culture medium including Hydroxychloroquine (20-50 µM) as a SARS-CoV-2 blocker for 20 minutes. Then, treated wells were inoculated with SARS-CoV-2 Spike protein pseudotyped VSV-ΔG (VSV-ΔG-Sdel21). 48 hours of post infection, the plate was scanned with Axio Observer Fluorescence Microscope (Zeiss).

Immunocytochemistry (ICC)

Indirect immunofluorescence analysis was performed for cellular Spike protein and ACE-2 protein detection. Vero E6 cells which are infected with Spike pseudotyped VSV-ΔG virus were fixed with %4 Paraformaldehyde (PFA) and stained with anti-Spike antibody (SinoBiological 2019-nCoV Spike S1 Rabbit mAb, 40150-R007) (1:80 dilution), then incubated with 1:1000 diluted Alexa Fluor 568-conjugated goat anti-rabbit IgG secondary antibody. ACE-2 protein was detected with anti-hAce2 (R&D Human Ace2, AF933) (1:120 dilution), incubated with Alexa Fluor 568-conjugated anti-goat IgG secondary antibody (1:500 dilution).

Western Blot (WB)

Western Blot analysis was performed to validate SARS-CoV-2 Spike protein expression in HEK293T cells. Total protein was subjected to SDS-PAGE followed by immunoblotting to nitrocellulose membranes (250 mA for 1.15 hours). Rabbit SARS-CoV-2 (2019-nCoV) Spike S1 monoclonal antibody (40150-R007, Sino-Biological) and a horseradish peroxidase-conjugated secondary antibody (1/10,000) were used for detection. Antibodies were used at 1/500 for Spike S1 subunit and at 1/10,000 for β-actin.

Pseudovirus based neutralization assay

Serum samples were diluted in DMEM-5 (reduced serum media; 5% FBS, 1% L-glutamine, 1x MEM NEAA) from 1:30 to 1:2430. SARS-CoV-2 Spike pseudotyped VSV-ΔG virus was diluted in DMEM-5 to a final concentration of 10^{-4} TU/ml. 100 µl diluted serum and 50 µl diluted virus were mixed in a 96 well plate and incubated in 37^0C incubator for 1 hr. Then, serum-virus mixture was added to Vero E6 adherent cells (80% confluent) and incubated in a 5% (v/v) CO_2, 37 °C incubator for 2 days. Cells were fixed with 4% PFA and analyzed by using Tecan plate reader (Infinite M200 Pro, Life Science). The GFP fluorescence intensity was microscopically detected at 48 hours post-infection by Axio Observer Fluorescence Microscope (Zeiss).

Statistical Analysis

Data were analyzed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA) and expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to compare differences between groups, and P values are indicated by asterisks (****$p< 0.0001$, **$p < 0.01$, and *$p < 0.05$).

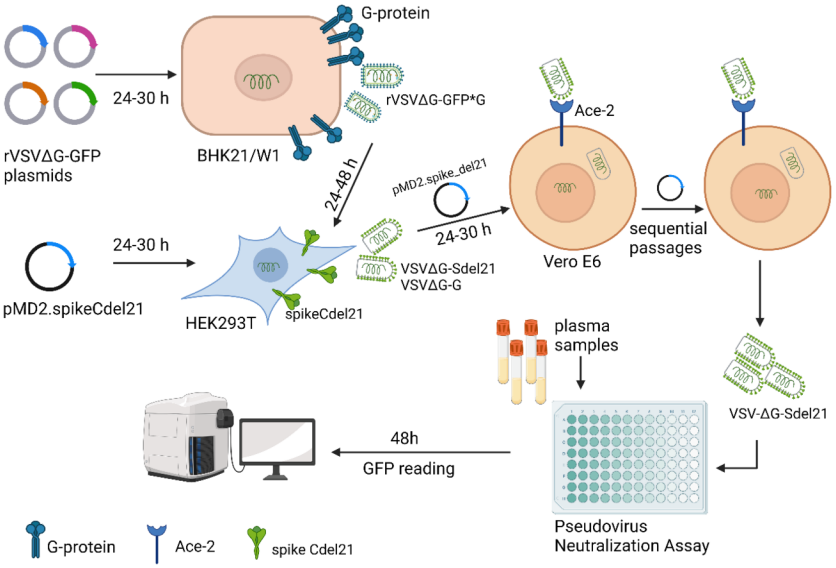

Figure 1. Generation of VSV pseudotyped viruses bearing truncated SARS-CoV-2 Spike proteins. **(A)** Preparation of rVSV-ΔG-G* in BHK21/W1 cell line. **(B)** Packaging of rVSV-ΔG-Sdel21 in HEK293T cell line. **(C)** Infection of pseudovirus carrying GFP reporter and sequential passaging of pseudovirus to eliminate parental virus (rVSV-ΔG-G*) using plasmids encoding Sdel21 and T7 polymerase transfected Vero E6. **(D)**SARS-CoV-2 Spike pseudovirus neutralization assay using human convalescent plasma samples and GFP signal reading.

RESULTS

G-protein deficient VSV bearing C-terminal 21 aminoacid truncated Spike protein were packaged in HEK293T cell line (**Figure 1**). To fully eliminate ancestral VSV-G, Spike pseudoviruses were serially passaged in pmD2.SpikeCdel21 transfected Vero E6 cells.

Hydroxychloroquine (HCQ) effectively inhibits viral entry and has been proposed for prophylaxis/treatment of SARS-CoV-2 infection. Therefore, HCQ was used to display inhibition of pseudovirus entry and construction of reliable pseudovirus assays. Harvested VSV-ΔG-Sdel21 pseudotyped virus were analyzed for viral entry using HCQ (20-50 μM). Virus infectivity was decreased with increasing concentration of hydroxychloroquine based on percentage of GFP-positive cell**(Figure 2A and 2B)** . The number of GFP-positive cells in VSV-ΔG-Sdel21-infected Vero E6 cells were significantly reduced from 30% to 10% in highest concentration of HCQ compared to positive control ($p<0.0001$). Representative image of positive control was given in Figure 2S.

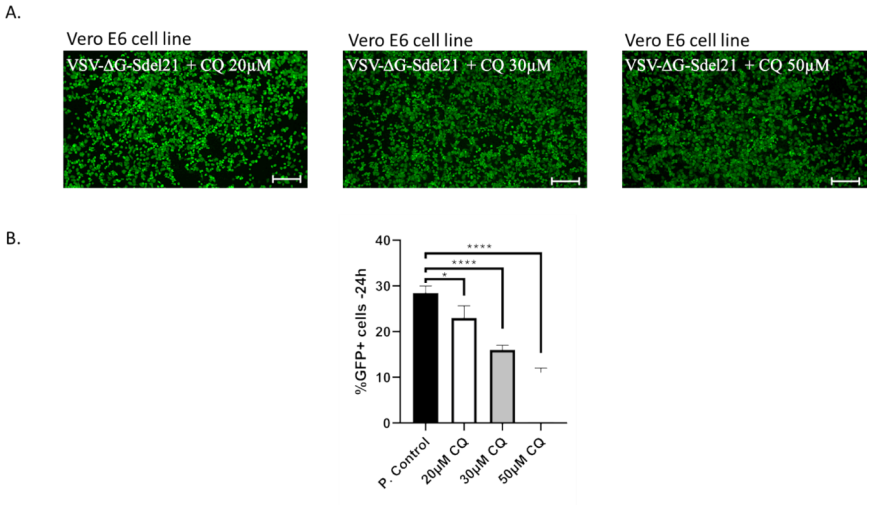

Figure 2. Viral entry assay in Vero E6 cell line. **(A)**Vero-E6 cells were inoculated with VSV-ΔG-Sdel21 pseudotyped virus. Cells were treated with hydroxychloroquine at 20 min before inoculation. After 24h, GFP signal detected under fluorescence microscope is shown in representative cellular images. **(B)** For quantification, GFP signal from 25 images per sample was analyzed by using Image J at 24h of post-inoculation. Values are presented as % of total population. Scale bar: 100μm. Data are represented as means of ±S.D. of at least three independent experiments. *p < 0.05; ****p < 0.0001 - using One-Way ANOVA with Bonferroni's post-hoc test.

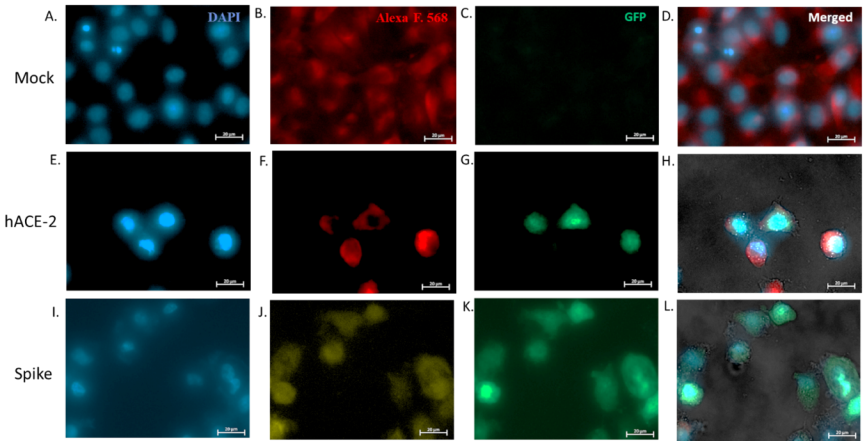

Figure 3. Colocalization of pseudovirus with hACE-2 receptor or SARS-CoV-2 Spike protein in Vero E6 cell line. Cells were incubated with anti-SARS-CoV-2 Spike, human anti-ACE2 and DAPI. **(A–D)**Mock-treated VeroE6 cells with hACE2 staining. **(E-H)**VSV-ΔG-Sdel21 infected Vero E6 cells with hACE2 staining. **(I-L)**VSV-ΔG-Sdel21 infected Vero E6 cells with SARS-CoV-2 Spike staining. Yellow color shows viral S protein (**J**), and red colors represents human ACE2 (**B, F**). Scale bars, 20 μm. Data in**A–L** are representative images of two independent experiments.

To validate Spike and ACE2 protein expression of infected cells, immunostaining was performed using VSV-ΔG-Sdel21 pseudotyped virus. Meanwhile, colocalization of VSV-ΔG-Sdel21 and human ACE2 receptor was investigated. **(Figure 3E-H)** . Co-localization was not found in mock-treated hACE2 **(Figure 3A–D),** indicating that SARS-CoV-2 uses human ACE2 as a receptor for entry. In addition, colocalization

of VSV-ΔG-Sdel21 and Spike protein confirmed the existence of Spike as a surface glycoprotein in our VSV-ΔG-Sdel21 pseudotyped particles

(Figure 3I-L).

The convalescent plasma samples were analyzed with VSV-ΔG bearing truncated SARS-CoV-2 Spike protein (VSV-ΔG-Sdel21). Spike pseudotyped viruses acquire the host spectrum of coronavirus and a rapid read out of the results is likely by the transfer of the *GFP* gene as a measure of infection. Administration of neutralizing antibodies against Spike pseudotyped VSV-ΔG virus reduce the number of GFP-positive cells. Neutralization activity of plasma was calculated based on GFP signal. Neutralization activities of plasma samples were depicted in

Figure 4 . With this assay, samples 2, 3, 4, 6, 7, 8, 9, 10, and 11 showed high neutralization activity with 50% inhibition above 1:270 dilutions.

To confirm data obtained by pseudovirus neutralization assay, we performed Plaque Reduction Assay (PRA) against SARS-CoV-2 Wuhan variant by using the same convalescent plasma samples. PRA is the gold standard phenotypic method to determine antiviral activity of plasma samples. This assay relies on quantifying the titer of neutralizing antibody through the number of plaques forming units (pfu) generated in a monolayer of virus-infected cells. Isolated SARS-CoV-2 was used to infect Vero E6 cells in the presence of decreasing concentrations of convalescent plasma and the 50% plaque reduction titer was determined. Several samples (# 7, 8, 9, and 11) showed high neutralization activity over a PRA50 of 1:320 (**Table 2**).

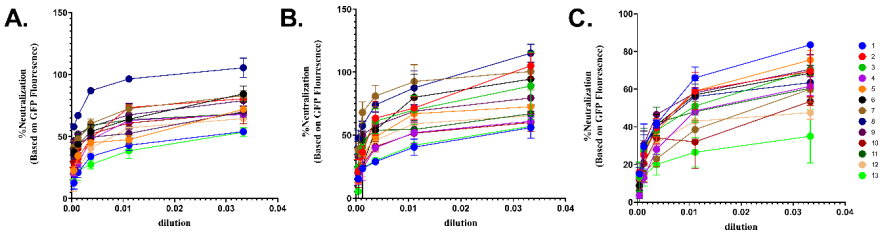

Figure 4. In vitro pseudovirus neutralization activity of human plasma samples (1-13) against SARS-CoV-2 Spike variants; *Wuhan*(**A**), *Alpha* (**B**), *Beta* (**C**). Neutralization curves of human convalescent plasma samples against pseudotyped SARS-CoV-2 variants; (**A**) VSV-ΔG-Sdel21 *Wuhan*type, (**B**) VSV-ΔG-Sdel21 *Alpha* variant carrying ΔH69-V70, N501Y, D614G, ΔY144 and (**C**) VSV-ΔG-Sdel21 *Beta* variant carrying E484K, N501Y, D614G, K417N mutations were measured as GFP signal in Vero E6 cells, and the percentage of neutralization was calculated. Data are represented as means of ±S.D. of at least two independent experiments.

Table 2. PRA Titers and Vaccination Profile of Human Plasma Samples

#Plasma samples	Vaccination	Neutralization titer by plaque assay
1	Sinovac, 2 dose	1:10
2	Sinovac, 2 dose	1:20
3	Sinovac, 2 dose	1:20
4	Sinovac, 2 dose; BioNTech, 1 dose	1:80
5	Sinovac, 2 dose; BioNTech, 1 dose	1:80
6	Sinovac, 2 dose; BioNTech, 1 dose	1:80
7	Sinovac, 2 dose	1:320
8	Sinovac, 2 dose; BioNTech, 1 dose	1:1280
9	BioNTech, 2 dose	1:640
10	BioNTech, 2 dose	1:160
11	BioNTech, 2 dose	1:320

#Plasma samples	Vaccination	Neutralization titer by plaque assay
12	Sinovac, 2 dose	1:40
13	Sinovac, 2 dose	1:10
14	Healthy control	0
15	Healthy control	0

In **Figure 5** , pseudovirus neutralization assay results were plotted against PRA results (*Wuhan* type) to compare their reliability. Plots showed linearity with a measure of certainty of $R^2 = 0.7$ (p= 0.0006) for pseudovirus neutralization against PRA with isolated authentic virus (**Figure 5A)** . The measure of certainty (R^2) was decreased for pseudovirus bearing Spike protein of *Alpha* and *Beta*variants against the PRA with *Wuhan* strain (**Figure 5B and 5C**). Linearity test of pseudovirus bearing Spike protein of*Alpha* variant was less significant for the comparison of pseudovirus bearing Spike protein of *Wuhan* strain versus PRA ($R^2 = 0.31$, p= 0.048). In general, the pseudovirus neutralization assay overestimated the samples with low neutralizing activities determined by PRA. It might be less reliable for plasma samples with low neutralizing capacity.

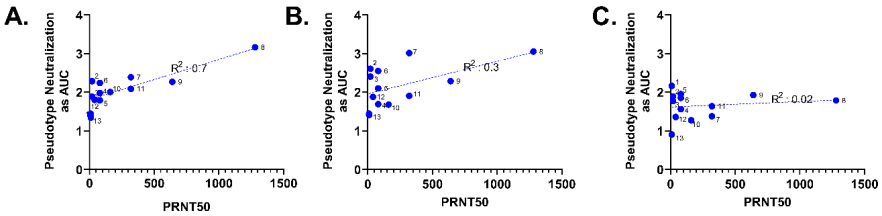

Figure 5 . **Comparison of pseudovirus neutralization assays with the plaque reduction assay.** Pseudovirus neutralization assays of *Wuhan* **(A)** , *Alpha* **(B)** and*Beta* variants **(C)** were plotted against the virus neutralization activities determined by PRA with authentic *Wuhan*virus. The R^2 value indicates the certainty of the values to be at the trend line.

DISCUSSION

Viral epidemics can easily spread around the worldas recently witnessed for COVID-19 epidemic. Efficient viral tools are necessary for developing vaccines or therapeutic drugs; however, handling infectious SARS-CoV-2 requires biosafety level-3 (BSL-3) facilities, which limits the research and development of therapeutic approaches. Therefore, developing safer and easier viral assays for SARS-CoV-2 became crucial to grapple with the viral epidemic in a widespread manner. The pseudovirus system is a promising approach to evaluate viral inhibitors and neutralizing antibodies against SARS-CoV-2, in convalescent plasma (Chen and Zhang, 2021). Although, both Lentivirus and Vesicular Stomatitis Virus (VSV) are commonly used as pseudovirus systems, the pseudotyped virus titer obtained in the VSV-ΔG system is generally higher than in the retrovirus systems (Li et al., 2018).

VSV is a glycoprotein (G)-enveloped negative-stranded RNA virus that infects a wide range of animals and rarely humans causing mild symptoms of flu (Rodriguez, 2002; Tani et al., 2011). VSV can effectively integrate and express irrelevant transmembrane proteins onto the surfaces of recombinant virus particles. In addition, their easily modifiable small genome (11 kb) and abundant replication in a wide range of cell lines have favored the use of VSV in pseudovirus systems (Almahboub et al., 2020). On the other hand, VSV pseudovirus system might not reflect the density and distribution of Spike protein, because viral surface geometry of the authentic spherical SARS-CoV-2 virus is different from bullet shape VSV (Chen and Zhang, 2021).

Besides, there may be residual VSV-ΔG-G* virus interfere with the pseudovirus due to its packaging process in the generation of pseudovirus. Parental VSV-G may cause additional infection apart from VSV-ΔG-

Spike pseudovirus and lead to false-positive results (Li et al., 2018). To solve this issue, serial passaging during pseudovirus generation and antibodies against G protein could be utilized (Condor Capcha et al., 2021; Yahalom-Ronen et al., 2020). Yahalom-Ronen et al. showed that at least 5 passages of recombinant VSV-ΔG-Spike virus ensure elimination of residual VSV-G. They also reported that sequential passaging of pseudotyped virus result in increased prevalence of Spike protein structures per single particle (Yahalom-Ronen et al., 2020). Therefore, in wet-laboratory processes, we applied 6 serial passaging of VSV-ΔG-Spike pseudovirus with Spike protein in Vero E6 cells to remove virions bearing G protein.

In advance of pseudovirus neutralization assay, we showed SARS-CoV-2 Spike based pseudovirus entry by using HCQ, a blocker for SARS-CoV-2 (Yuan et al., 2022). Increasing concentration of HCQ led to a decrease in GFP signal in Vero E6 cells. GFP fluorescence signal was utilized as a measurement of infectivity encoded by the main VSV plasmid. GFP signaling is a rapid and inexpensive tool, allowing to follow by live imaging systems and flow cytometry. There are many alternative reporters described in literature for the pseudovirus system such as Red Fluorescence Protein (RFP/dsRed), firefly Luciferase (fLuc), and secreted embryonic alkaline phosphatase (SEAP). Although both fLuc and SEAP require additional steps and reagents, fLuc is the most preferred reporter for both VSV and LV pseudovirus generation in literature (Donofrio et al., 2021; Nie et al., 2020). Several studies demonstrated slower kinetics and sensitivity for fluorescent proteins (GFP or DsRed) when compared to Firefly luciferases (Neefjes et al., 2021). However, GFP reporter provides robust expression in the cell and ease to follow both generation and infectivity processes. We used the intensity of GFP fluorescence to determine VSV titer and inhibition. On the other hand, it might lead to more accurate results, if both GFP and fLuc reporter encoding plasmid backbone are used for pseudovirus generation (Crawford et al., 2020).

The infection model consisting of Vero E6 cells and VSV-ΔG-Spike can mimic the entry of authentic SARS-CoV-2. The serum neutralizing titer of vaccinated convalescent patients measured by the VSV-ΔG-Spike pseudovirus assay has a good correlation with that measured by the authentic SARS-CoV-2 PRA assay. In addition, the pseudovirus assay is safer and time-efficient than the wild type SARS-CoV-2 PRA assay. More importantly, this method can be used to determine the neutralization titer of serum against the SARS-CoV-2 variants. In our pseudovirus study, *Alpha* and *Beta* variants of Spike protein were constructed and tested against human convalescent plasma samples listed in **Table 1** . It is shown that the pseudovirus assay based on the VSV-ΔG system was successful in discrimination of different SARS-CoV-2 variants. In consistence with literature, pseudovirus neutralization assay exaggerated the potency of samples with low neutrality activity, but increased the inhibition limit, enabling the identification of samples with high neutrality potency (von Rhein et al., 2021). Each vaccinated human convalescent serum sample was assayed for neutralization against *Alpha* (B.1.1.7), *Beta* (B.1.351), and *Wuhan* (Wild type) viruses. The pseudovirus neutralization assay showed no loss of neutralizing activity against Spike *Alpha*variant, whereas every sample lost activity against Spike *Beta*variant. Overall, the neutralizing activity against *Alpha* Spike pseudotyped virus was essentially unchanged, but significantly lower against *Beta* Spike pseudotyped virus. The main contributing factor is suggested to be E484K mutation in *Beta* variant which provides neutralization resistance of the virus. Studies indicate that this mutation in receptor binding motif is in an immunodominant epitope of Spike protein (Garcia-Beltran et al., 2021; Wang et al., 2021).

When Sinovac and BioNTech-Pfizer mRNA vaccine BNT162b2 were compared, BioNTech-vaccinated plasma samples were highly neutralizing according to PRA and pseudovirus neutralization assay. In consistent with literature, the human plasma had slightly reduced but overall, largely preserved neutralizing titers against the *Alpha* variant pseudovirus (Muik et al., 2021). The largely preserved neutralization of pseudovirus bearing the *Alpha* Spike by BNT162b2-immune plasma makes it unlikely that the *Beta* variant virus will escape BNT162b2-mediated protection. However, pseudovirus neutralization assay showed a substantial drop-in neutralization activity against the*Beta* variant, consistent with conclusions being reached by others (Hoffmann et al., 2021; Wang et al., 2021).

Even though pseudoviruses only contain the envelope proteins of the authentic virus, pseudovirus-based assays have demonstrated a good correlation with the infectious virusbased assay. Besides, the pseudovirus-

based neutralization assays are usually highhroughput procedures requiring less amounts of serum samples (Sanders, 2002). Our pseudovirus neutralization assay can be used to evaluate the neutralization potency of vaccines or antibodies. Taken together, this convenient and reliable pseudovirus system based on a VSV packaging system could be greatly beneficial for developing SARS-CoV-2 vaccines and therapeutic drugs as well as for testing the inhibition potency of convalescent plasma samples.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Author contribution

GCT conducted the experiments, performed the statistical analysis, wrote, and edited the original draft. ANC and YTT participated in experiments. OK and SC reviewed the manuscript. Study concept and design: YTT, EY, NA, DS, GE, OD, MAK, BM, GA, FT, FC, OE, KM, OK, SC. All authors contributed to and have approved the final version of the manuscript.

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Figure 1. Generation of VSV pseudotyped viruses bearing truncated SARS-CoV-2 Spike proteins. **(A)** Preparation of rVSV-ΔG-G* in BHK21/W1 cell line. **(B)** Packaging of rVSV-ΔG-Sdel21 in HEK293T cell line. **(C)** Infection of pseudovirus carrying GFP reporter and sequential passaging of pseudovirus to eliminate parental virus (rVSV-ΔG-G*) using plasmids encoding Sdel21 and T7 polymerase transfected Vero E6. **(D)**SARS-CoV-2 Spike pseudovirus neutralization assay using human convalescent plasma samples and GFP signal reading.

Figure 2. Viral entry assay in Vero E6 cell line. **(A)**Vero-E6 cells were inoculated with VSV-ΔG-Sdel21 pseudotyped virus. Cells were treated with hydroxychloroquine at 20 min before inoculation. After 24h, GFP signal detected under fluorescence microscope is shown in representative cellular images. **(B)** For quantification, GFP signal from 25 images per sample was analyzed by using Image J at 24h of post-inoculation. Values are presented as % of total population. Scale bar: 100μm. Data are represented as

means of ±S.D. of at least three independent experiments. *p < 0.05; ****p < 0.0001 - using One-Way ANOVA with Bonferroni's post-hoc test.

Figure 3. Colocalization of pseudovirus with hACE-2 receptor or SARS-CoV-2 Spike protein in Vero E6 cell line. Cells were incubated with anti-SARS-CoV-2 Spike, human anti-ACE2 and DAPI. **(A–D)**Mock-treated VeroE6 cells with hACE2 staining. (**E-H)**VSV-ΔG-Sdel21 infected Vero E6 cells with hACE2 staining. **(I-L)**VSV-ΔG-Sdel21 infected Vero E6 cells with SARS-CoV-2 Spike staining. Yellow color shows viral S protein (**J**), and red colors represents human ACE2 (**B, F**). Scale bars, 20 μm. Data in**A**–**L** are representative images of two independent experiments.

Figure 4. In vitro pseudovirus neutralization activity of human plasma samples (1-13) against SARS-CoV-2 Spike variants; *Wuhan*(**A**), *Alpha* (**B**), *Beta* (**C**). Neutralization curves of human convalescent plasma samples against pseudotyped SARS-CoV-2 variants; (**A)** VSV-ΔG-Sdel21 *Wuhan*type, (**B)** VSV-ΔG-Sdel21 *Alpha* variant carrying ΔH69-V70, N501Y, D614G, ΔY144 and (**C)** VSV-ΔG-Sdel21 *Beta* variant carrying E484K, N501Y, D614G, K417N mutations were measured as GFP signal in Vero E6 cells, and the percentage of neutralization was calculated. Data are represented as means of ±S.D. of at least two independent experiments.

Figure 5 . Comparison of pseudovirus neutralization assays with the plaque reduction assay. Pseudovirus neutralization assays of *Wuhan* **(A)** , *Alpha* **(B)** and*Beta* variants **(C)** were plotted against the virus neutralization activities determined by PRA with authentic *Wuhan*virus. The R^2 value indicates the certainty of the values to be at the trend line.

Table 1. Vaccination status of human plasma samples used for neutralization assays

Table 2. PRA Titers and Vaccination Profile of Human Plasma Samples

Figure 1S. Spike expressing construct map

Figure S2 . Positive control images of viral entry assay in Vero E6 cell line. Vero-E6 cells were inoculated with VSV-ΔG-Sdel21 pseudotyped particles. After 24 h, the GFP signal was observed using Axio Observer Fluorescence Microscope. The representative cellular images were given.

Table S1. The sequences of the mutation primers