RESEARCH ARTICLE

SARS-CoV-2 Neutralization Assay System using Pseudo-lentivirus

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Abstract

BACKGROUND: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infects humans' lower respiratory tracts and causes coronavirus disease-2019 (COVID-19). Neutralizing antibodies is one of the adaptive immune system responses that can reduce SARS-CoV-2 infection. This study aimed to develop a SARS-CoV-2 neutralization assay system using pseudo-lentivirus.

METHODS: The plasmid used for pseudo-lentivirus production was characterized using restriction analysis. The gene encoding for SARS-CoV-2 spike protein was confirmed using sequencing. The transfection pseudo-lentivirus optimal condition was determined by choosing the transfection reagents and adding centrifugation step. Optimal pseudo-lentivirus infection was analysed using fluorescent assay and luciferase assay. The optimal condition of pseudo-lentivirus infection was determined by the target cell type and the number of pseudo-lentiviruses used for neutralization test. SARS-CoV-2 pseudo-lentivirus was used to detect neutralizing antibodies from serum samples.

RESULTS: The plasmid used for pseudo-lentivirus production was characterized and confirmed to have no mutations. Lipofectamine 2000 reagent generated pseudo-lentivirus with a higher ability to infect target cells, as indicated by a percentage green fluorescent protein (GFP) of 12.68%. Pseudo-lentivirus centrifuged obtained more stable results in luciferase expression. Optimal pseudo-lentivirus infection conditions were obtained using puromycin-selected HEK 293T-ACE2 cells as target cells. The number of pseudo-lentiviruses used in the neutralization assay system was multiplicity of infection (MOI) 0.075. Serum A samples with a 1:10 dilution had the highest neutralizing antibody activity.

CONCLUSION: This study shows that SARS-CoV-2 neutralization assay system using pseudo-lentivirus successfully detected neutralizing antibodies in human serum, which were indicated by a decrease in the percentage of pseudo-lentivirus infections.

KEYWORDS: COVID-19, neutralizing antibody, neutralization assay, pseudo-lentivirus, SARS-COV-2

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Introduction

Coronavirus disease-2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which infect humans' lower respiratory tracts and cause severe illness.(1,2) As the consequence, human host immune system fights back with several strategies, such as using adaptive immune system. The production of neutralizing antibodies is one of the adaptive immune system responses to SARS-CoV-2 infection.(3) To reduce viral infections, neutralizing antibodies prevent viruses from binding to

human cell receptors.(4) Neutralizing antibodies can also be formed as a result of the vaccine's reaction.(5) Hence, it is critical to detect and examine the function of neutralizing antibodies. The gold standard for detecting neutralizing antibody activities is virus-neutralization assay.(1)

However, SARS-CoV-2 is highly infectious and pathogenic, and handling the virus must be done in a biosafety level (BSL)-3 laboratory.(3) The limited number of BSL-3 laboratories may cause delays in vaccine development and COVID-19 treatment. Therefore, alternative approaches to studying this virus are required.(6)

Pseudoviruses are recombinant viruses that consist of a viral core or backbone and surface proteins obtained from different viruses.(6,7) Pseudovirus represents a lower biological safety risk than natural pathogenic viruses; thus, it can be conducted in a BSL-2 laboratory. Pseudoviruses have been widely used in the research of other pathogenic viruses, including Middle East respiratory syndrome (MERS), severe acute respiratory syndrome (SARS), ebola, hanta, and the Seoul virus.(7)

Previous studies on neutralization assay using lentiviral pseudotypes have shown that the assay has a sensitivity rate of 85.7% and a specificity rate of 100% compared to microneutralization (MN) assay using SARS-CoV-2.(8) Similar results were obtained using 300 serum samples that have shown neutralization assay using lentiviral pseudotypes has 85.9% sensitivity and 100% specificity compared to MN assay.(10)

Pseudovirus-based neutralization assay is more advantageous because the assay can be carried out in a BSL-2 laboratory facility and uses fewer resources than the MN assay. This assay is a safer method for treating highly contagious pathogens such as SARS-CoV-2.(9) Therefore, a lentiviral pseudotypes neutralization assay is a good alternative for further studies on SARS-CoV-2.(10)

In this study, we developed a SARS-CoV-2 neutralization assay system using pseudo-lentivirus according to the protocol (11) with some modification. The modification of the previous study includes optimization and also utilization of D614G Spike mutant. We obtained pseudo-lentivirus bearing the SARS-CoV-2 spike protein that has a mutation on D614G that has an effect on higher infectivity.(12,13) In this study, we used the third-generation lentiviral packaging system, to produce pseudo-lentivirus. We explored new plasmid combinations to increase the infectivity of pseudo-lentivirus. The SARS-CoV-2 pseudo-lentivirus system was optimized and then used to detect neutralizing antibodies in serum samples, as initial testing.

Methods

Cells and Samples

Human embryonic kidney (HEK293T) cells and HEK293T expressing hACE2 (HEK293T-ACE2) cells were kindly provided by Dr. Yoshiharu Matsuura from Osaka University, Osaka, Japan.The cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, New York, NY, USA) and 1% penicillin-streptomycin-amphotericin B Mixture (Lonza, Basel, Switzerland) in 5% CO_2 incubator at 37°C. The selection of HEK293T-ACE2 cells was cultured under the same conditions with an additional 1 µg/mL puromycin dihydrochloride (Sigma-Aldrich) to the medium for 14 days prior to use.

Human serum samples were collected from donors with written informed consent. The donor was in healthy condition and has already been vaccinated with the COVID-19 vaccine in Indonesia. The negative control serum was obtained from commercial human serum (Sigma-Aldrich). All serum samples were filtered using membrane filter PES 0.22 µm and heat-inactivated at 56°C for 1 hour. This study was approved by the Medical Research Ethics Committee of Universitas Jenderal Achmad Yani, Indonesia (No. 164/ KEPK/ FITKES-UNJANI/VIII/2022).

Plasmids

The third-generation lentiviral packaging system which consists of three component plasmids and available in Addgene (*https://www.addgene.org*) was used. The packaging plasmids were pMDLg-pRRE (#12251) and pRSV-Rev (#12253). The transfer plasmid was pFUGW-Pol2-ffLuc2-eGFP (#71394) which encodes reporter eGFP and firefly luciferase. The envelope plasmid was HDM-SARS2-Spike-del21-D614G (#158762) which encodes SARS-CoV-2 spike protein D614G mutation. All plasmids were isolated using GeneJET Plasmid Midiprep Kit (Thermo-Fisher, Waltham, MA, USA). DNA plasmids characterized with restriction analysis and fragment size were compared in gel agarose electrophoresis 1%. The gene encoding for SARS-CoV-2 spike protein in plasmid HDM-SARS2-Spike-del21-D614G was confirmed using sequencing in Macrogen, Singapore, and the sequence alignment was analysed using BioEdit Sequence Alignment Editor version 7.2 (Informer Technologies Inc, Los Angeles, CA, USA).

Pseudo-lentivirus Transfection

The HEK293T cells were seeded at a density of 3.4×10^5 cells per well on cell culture plate 6-well and maintained for 24 hours until the cell density reached 80-90% confluency. Next, cells were then transfected with the plasmid DNA to produce lentiviruses. The transfection was performed using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) or Lipofectamine 3000 (Invitrogen) according to manufacturer's instructions. The fresh medium of 1.5 mL was added 5 hours post-transfection, and cells were incubated for 72 hours in a 5% CO_2 incubator at 37°C. The cell culture supernatant was collected, pooled, and centrifuged at 1,000 rpm for 10 min at 4°C. The pseudo-lentivirus was collected, aliquoted, and stored at -80°C until used.

Pseudo-lentivirus Infection

Analysis of pseudo-lentivirus infection were performed by detecting the enhanced green fluorescent protein (eGFP) expression and luciferase expression. For the eGFP detection, the HEK 293T-ACE2 cells were seeded at a density of 2.5×10^4 cells per well in 8-well cell culture slide coated with poly-L-lysine (Sigma-Aldrich) and then incubated for 48 hours. The cells in each well were infected with 400 µL pseudo-lentiviruses and incubated for 48 hours. Cell cultured slide was fixed using 3.7% formaldehyde, stained with 1 µg/mL Hoechst, and then mounted. The eGFP expression was detected using Confocal Laser Scanning Microscope Olympus FV-1200 (Olympus, Tokyo, Japan).

For luciferase assay, the HEK 293T-ACE2 cells were seeded at a density of 1.2×10^4 cells per well in black bottom 96-well plates 24 hours before infection. The cells in each well were then infected with pseudo-lentiviruses at various multiplicity of infection (MOI). Luciferase activity was detected 48 hours post-infection using Steady-Glo® Luciferase Assay System (Promega, Madison, WI, USA) following the manufacturer's instructions.

Pseudo-lentivirus Neutralization Assay

HEK 293T-ACE2 cells were seeded at a density of 1.2×10^4 cells per well in black bottom 96-well plates 24 h before infection. The human serum was diluted serially and added with pseudo-lentivirus MOI of 0.075. The mixed suspension was incubated at 37°C for 1 hour. The suspension was added to each well and incubated for 48 hours in a 5% CO_2 incubator at 37°C. The luciferase activity was detected 48 hours post-infection using Steady-Glo® Luciferase Assay System (Promega) following the manufacturer's instructions. The activity of neutralizing antibodies was indicated by decreasing luciferase expression.

Statistical Analysis

Collected data are analyzed by ANOVA Test and Tukey Post-Hoc to determine the significance difference with the aid of SPSS statistical software version 22 (IBM Corporation, Armonk, NY, USA).

Results

Plasmids Characterization and Confirmation

The restriction analysis was used to characterize the plasmids used in this study. Restriction analysis of pFUGW-Pol2-ffLuc2-eGFP plasmid using EcoRI and BamHI showed two fragments with experimental size of 2,779 and 5,905 bp. The pMDLg-pRRE plasmid cut using AseI showed two fragments with experimental size of 917 and 6,452 bp. Restriction of HDM-SARS2-Spike-del21-D614G plasmid using HindIII showed two fragments with experimental size of 3,174 and 5,649 bp. The restriction analysis of the pRSV-Rev plasmid using BamHI revealed a fragment with experimental size of 4,731 bp (Figure 1). These results were aligned with the theoretical size (Table 1).

The gene encoding for SARS-CoV-2 spike protein in HDM-SARS2-Spike-del21-D614G plasmid was confirmed by sequencing method. Confirmation was performed to ensure that no mutations occurred. The sequencing results were then aligned with the provider's original sequence (Addgene) and analyzed with the BioEdit software. The sequence alignment results confirmed that there was no mutation in the gene coding for the spike of SARS-CoV-2, resulting in 100% sequence alignment. Therefore, this plasmid can be used for pseudo-lentivirus production with the SARS-CoV-2 spike protein coding gene.

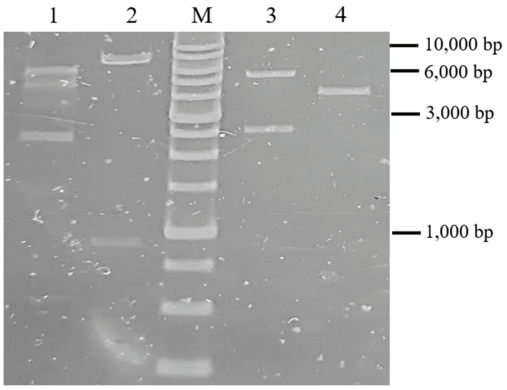

Figure 1. Restriction analysis results in agarose gel electropherogram. M: 1Kb DNA marker; 1: pFUGW-Pol2-ffLuc2-eGFP + EcoRI + BamHI; 2: pMDLg-pRRE + AseI; 3: HDM-SARS2-Spike-del21-D614G + HindIII; 4: pRSV-Rev + BamHI.

Table 1. Theoretical restriction fragment size analysis.

Plasmid	Theoretical Fragment Size (bp)
pFUGW-Pol2-ffLuc2-eGFP	6,604
	2,430
pMDLg-pRRE	7,957
	933
HDM-SARS2-Spike-del21-D614G	5,669
	2,647
pRSV-Rev	4,180

Determination of Pseudo-lentivirus Transfection Conditions

The ability of the pseudo-lentivirus to infect target cells was measured in this study to determine optimal pseudo-lentivirus transfection conditions. Detection of eGFP expression and luciferase expression were used to assess pseudo-lentivirus infection rate. The optimization of pseudo-lentivirus production was performed by comparing Lipofectamine 2000 and 3000 as transfection reagent. The produced virus was used to infect HEK293T-ACE2 cells and the eGFP expression level was detected using fluorescence microscope. The results showed that Lipofectamine 2000 in pseudo-lentivirus production generated 12.68% eGFP-positive cells, which was higher than the use of Lipofectamine 3000 (Figure 2A). The luciferase assay also showed that the relative light unit (RLU) value was higher when Lipofectamine 2000 was used in pseudo-lentivirus production, as compared to Lipofectamine 3000 (Figure 2B). This indicated higher pseudo-lentivirus produced using Lipofectamine 2000 as transfection reagent.

In early stage of this study, the luciferase assay result showed that the RLU in the first experiment was high, but the difference in RLU with the second experiment was very large (Figure 3A). This variable result suggested that the treatment during the harvest of pseudo-lentivirus have an impact to the RLU. Therefore, additional centrifugation was added in the next pseudo-lentivirus production. Pseudo-lentivirus produced with additional centrifugation step showed more consistent RLU between the two performed experiments (Figure 3B). This result indicated that the centrifugation step during pseudo-lentivirus harvest can separate the pseudo-lentivirus from the dead cells after the virus production, although lower RLU was observed.

Determination of Pseudo-lentivirus Infection Conditions

Optimization of pseudo-lentivirus infection conditions were performed by HEK293T-ACE2 selection using puromycin and various pseudo-lentivirus MOI. Selection of HEK293T-ACE2 cells using puromycin was performed because the generation of these cells employed puromycin resistance. The result showed that infection of puromycin-selected HEK 293T-ACE2 generated higher virus titer as compared to the non-selected HEK 293T-ACE2 cells (Figure 4).

Various MOIs were also tested to optimize the condition for neutralization test using pseudo-lentivirus. The MOI values used in this study were MOI 0.1; 0.075; 0.05; and 0.025. The results showed that the highest luciferase expression was obtained at MOI 0.075 and 0.05 but the standard deviation was lower at MOI 0.075 (Figure 5). Additionally, the luciferase expression was higher in puromycin-selected HEK 293T-ACE2, as observed based on the RLU (Figure 5). This data indicated that selection of HEK293T-ACE2 cells could eliminate the cells that did not express ACE2, therefore improving the infection of pseudo-lentiviruses. This condition was used for initial testing of pseudo-lentivirus in the neutralization assay.

SARS-CoV-2 Neutralization Assay using Pseudo-lentivirus

The neutralization assay was performed using serum from subjects that have received full COVID-19 vaccination. The result of this study showed that the system can detect neutralizing antibodies which was indicated by a decrease in the percentage of pseudo-lentivirus infection. However,

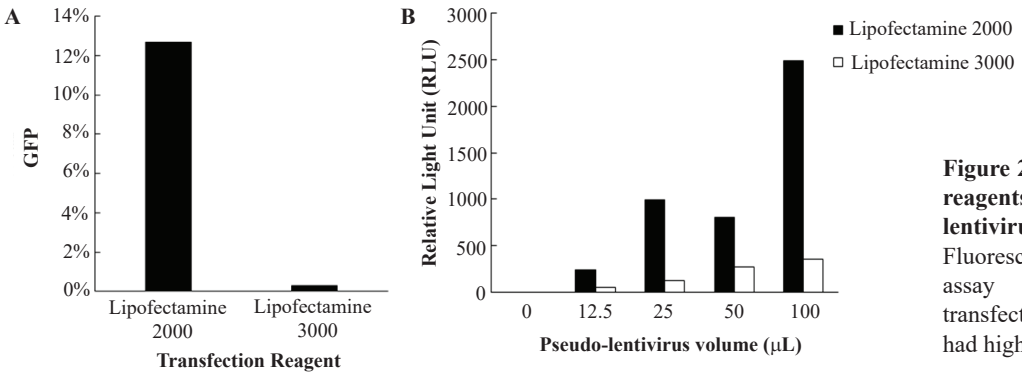

Figure 2. Comparison of transfection reagents to generate pseudo-lentiviruses with higher infectivity. A: Fluorescent assay results; B: Luciferase assay results. Pseudo-lentiviruses transfected with Lipofectamine 2000 had higher infectivity.

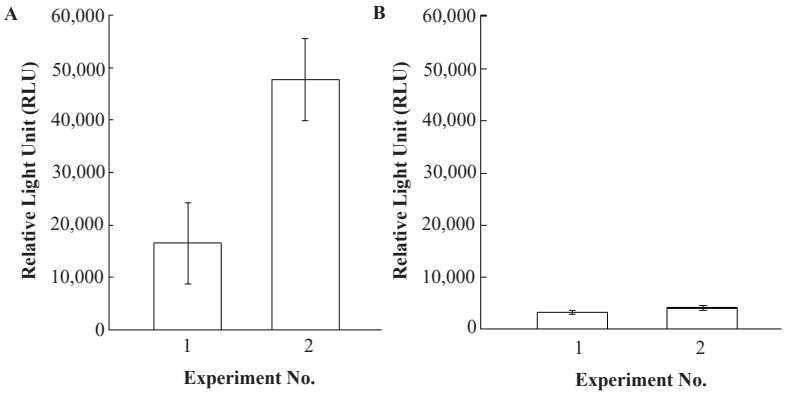

Figure 3. The impact of pseudo-lentivirus centrifugation on luciferase assay results. A: Pseudo-lentivirus produced without centrifugation; B: Pseudo-lentivirus produced with centrifugation. Pseudo-lentivirus centrifuged obtained more stable results than pseudo-lentivirus without centrifuged, but the expression of luciferase is quite low, thus efforts are needed to increase the expression of luciferase (data shown as mean±SD)

statistical analysis revealed that serum A at 1:10 dilution was significantly different from the negative control at 1:100 dilution with p=0.055 (Figure 6). This implied that serum A samples with a 1:10 dilution had the highest neutralizing antibody activity.

Discussion

The third generation of lentiviral packaging system was created to be safer because the packaging plasmids split into two plasmids, one encoded Rev and the others Gag and Pol. This separation aimed to reduce the chance of developing recombinant HIV *in vitro*.(7) The envelope plasmid HDM-SARS2-Spike-del21-D614G used in this study has been modified with 21 aa deletions at the C-terminal and has the DG614 mutation.(12) This mutation led to increased infectivity of SARS-CoV-2, which caused fast transmission and is commonly found in the early COVID-19 pandemic.(13)

An amino acid deletion at C-terminal of the SARS-CoV-2 spike protein also plays a role in enhancing the formation of pseudoviruses. In the C-terminal, there is a signal for retention of the endoplasmic reticulum, thus its removal will promote spike transfer to cell surface. Hence, it could increase the incorporation of the SARS-CoV-2 spike protein into viral particles such as lentiviruses and VSV. (14) Therefore, the plasmid used in this study is designed to obtain a high level of infectivity of SARS-CoV-2 in the cell target. The result of this study showed that all plasmids used was confirmed and it can be used to produce pseudo-lentiviruses.

Lipofectamine was a transfection reagent that creates cationic lipids.(15) In this study, the pseudo-lentivirus transfection reagent was determined using two types of transfection reagents, Lipofectamine 2000 and 3000. This aims to optimize transfection efficiency, particularly in difficult-to-transfect cell types.(16) This study showed that higher infectivity of SARS-CoV-2 pseudo-lentivirus were observed when the virus was produced using the Lipofectamine 2000 reagent. This finding was in line with the results of study that the Lipofectamine 2000 reagent was the most effective transfection reagent for transferring nucleic acids into eukaryotic cells.(17) Lipofectamine 2000 was also used in the development of a SARS-CoV-2 neutralization assay system based on a VSV pseudovirus packaging system.(18)

Improvement of the assay was also performed by centrifugation of harvested pseudo-lentivirus. One study

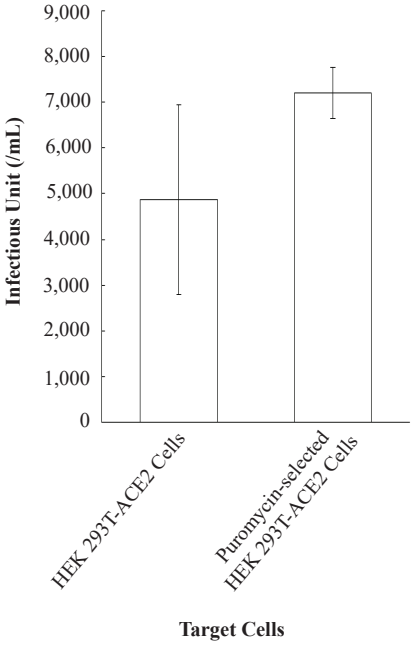

Figure 4. Comparison of the quantification of pseudo-lentivirus in HEK 293T-ACE2 cells and puromycin-selected HEK 293T-ACE2 cells. The puromycin-selected cells produced higher and more stable reporter expression (data shown as mean±SD of three independent experiments).

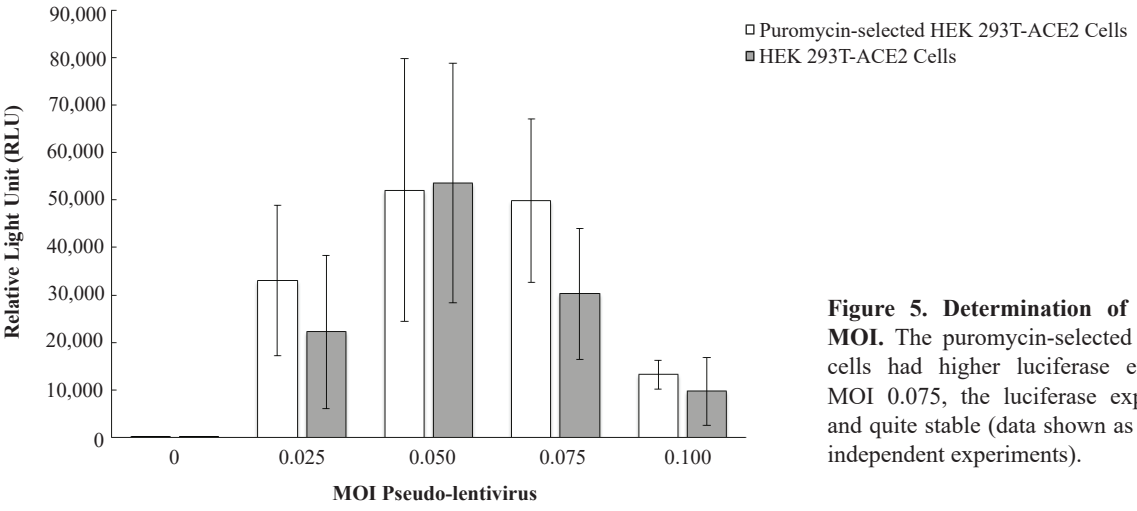

Figure 5. Determination of pseudo-lentivirus MOI. The puromycin-selected HEK 293T-ACE2 cells had higher luciferase expression, and at MOI 0.075, the luciferase expression was high and quite stable (data shown as mean±SD of three independent experiments).

showed that one of the important steps in pseudovirus transfection is centrifugation.(7) In the centrifugation step, virus will be separated from the carried over transfected cells. Transfected cells used for pseudo-lentivirus production also expressed luciferase and eGFP, which then contributed to the measured signals. Therefore, it was important to separate it in order to gain the solely virus. However, expression of luciferase was quite low and more investigation is needed on how to increase its expression.

In this study, we used HEK 293T-ACE2 as target cells for pseudo-lentivirus infection. HEK 293T-ACE2 cells were a modified HEK 293T cell line that expresses the receptor angiotensin-converting enzyme 2 (ACE2).(19) The results showed that the infectious unit number on puromycin-selected HEK 293T-ACE2 cells was higher and more stable than on HEK 293T-ACE2 cells without puromycin selection. This result was in line with previous research where puromycin selection increased the expression of ACE2.(20) Puromycin selection removed cells that did not express ACE2 due to the stable integration of ACE2 employed puromycin resistance as selection marker.

The result of this study showed highest expression of luciferase and more consistent result were obtained at MOI 0.075. The previous study that used VSV pseudovirus for a

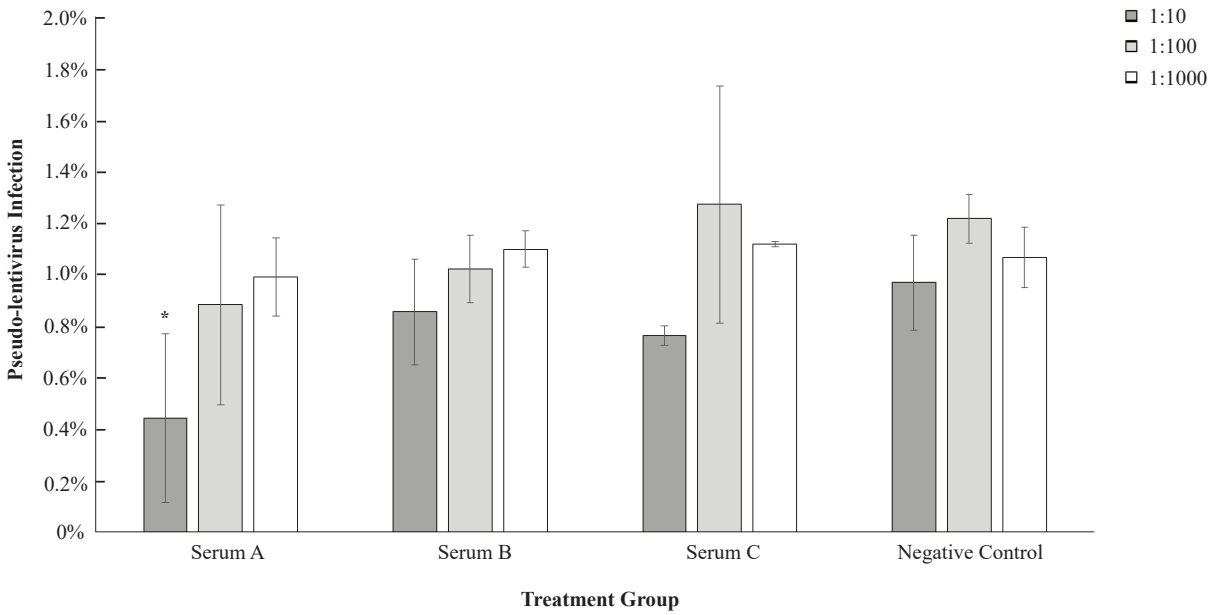

Figure 6. The results of the SARS-CoV-2 neutralization assay using pseudo-lentivirus. The system can detect neutralizing antibodies in human serum samples which indicated by a decrease in the percentage of pseudo-lentivirus infection (data shown as mean±SD of to to three independent experiments). *p-value<0.10, and compared to negative control at 1:100 serum dilution, tested with ANOVA and Tukey Pos-Hoc Test.

neutralization test against SARS-CoV-2 with baby hamster kidney (BHK) 21-ACE2 was carried out at the MOI range of 0.00625 to 0.2.(10) The study showed that the optimal MOI was obtained at MOI 0.05. A neutralization test against SARS-CoV-2 using VSV was also carried out using MOI 1 on Vero E6 target cells.(21) Thus, the determination of the different MOI values in each experiment was adjusted to the experimental conditions, such as the type of pseudovirus and the type of target cells.

The luciferase reporter gene carried in the pseudoviruses will be expressed if the virus infect the target cells, thus reporter expression can be measured using a luminometer after the cells are lysed.(22) This study showed that the pseudo-lentivirus system can detect the neutralizing antibodies in serum samples which was indicated by the decrease of virus infection. This result was in line with the study, which found that a decrease in luminescence indicates neutralizing activity of neutralizing antibodies in the serum samples tested.(3) The results showed that serum A at 1:10 dilution had the highest neutralizing antibodies compared to the negative control at 1:100 dilution based on statistical analysis. The lack of positive control serum which contained SARS-CoV-2 neutralizing antibodies that has been measured was the limitation of this study.

The developed and optimized pseudo-lentivirus system in this study can detect neutralizing antibodies. The optimum transfection was done using Lipofectamine 2000 to obtain higher infectivity and a centrifugation step at pseudovirus harvest was needed. Selection of HEK 293T-ACE2 cells using puromycin increased the gene reporter expression and the optimum number of pseudo-lentivirus was obtained at MOI 0.075.

Conclusion

SARS-CoV-2 neutralization assay system using pseudo-lentivirus was successfully developed to detect neutralizing antibodies in human serum. However, further research needs to be done, *i.e.*, neutralization assay using pseudo-lentivirus having the several variants of SARS-CoV-2 spike protein.

Acknowledgements

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Authors Contribution

AAt, EAGR, MIT, and DN were involved in planning and supervised the work. DVC, CO, and YM were aided in experimental design. AAm, AFS and KV performed the measurements, processed the experimental data, and performed the analysis. AAm, AAt and EAGR aided in interpreting the results and worked on the manuscript. All authors discussed the results and commented on the manuscript.

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