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Submission date: 02-Aug-2025 09:44PM (UTC+0700)
Submission ID: 2724119694
File name: Syarat_khusus_Silvia.pdf (429.09K)
Word count: 3284
Character count: 15866

IJTID. Volume 13 No 1 January-April 2025
Sinta 2 (Decree No: 105/E/KPT/2022)
e-ISSN: 2356-0991; p-ISSN:2085-1103
Available online at https://e-journal.unair.ac.id/IJTID

Original Article

IJTID
(INDONESIAN JOURNAL OF TROPICAL AND INFECTIOUS DISEASE)
Scientific Journal of Tropical and Infectious Disease

In Vitro Analysis of SARS-CoV-2 Variants that Caused Severe COVID-19 in the Elderly

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OPEN ACCESS

ARTICLE INFO

Received: December 11, 2024
Accepted: March 20, 2025
Published: April 30, 2025
Available online: April 30, 2025

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Keywords:
Plaque
SARS-CoV-2
Omicron
ImageJ
Elderly

Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) caused the global problem of respiratory disease from 2019 to 2024. One of the earliest variations in the SARS-CoV-2 S protein was the S D614G mutation. SARS-CoV-2 has several important variants, namely, Alpha, Beta, Gamma, Delta, and Omicron. Omicron is the variant that has caused severe health problems, some resulting in death, in the elderly. Omicron has further differentiated to some well-known variants, such as, BA.1, BA.2, BA.2.75, BA.5, BQ.1.1, and XBB.1. According to Japanese Government data, the number of citizens aged 65 years old and above reached 28.6% in 2021. From our previous experiment, antibodies of the elderly that have received four doses of mRNA vaccine still could not optimally neutralize Omicron BQ.1.1 and XBB.1. We aimed to analyze the plaque size of SARS-CoV-2 variants that caused severe COVID-19 in the elderly. SARS-CoV-2 variants were seeded in Vero E6-TMPRSS2 cell culture to create plaques. The resulting plaques were analyzed with ImageJ application to select solitary plaques and to determine plaque sizes. The size of BA.1 plaque was indifferent to BA.2 plaque. The plaque area comparison result was as follows, BA.1/BA.2<BA.5<BA.2.75<BQ.1.1<XBB.1. The plaque sizes of Omicron BQ.1.1 and XBB.1 were bigger that those of Omicron BA.1 and BA.2. The plaque sizes of all Omicron variants were smaller than those of the previous variants, S D614G and Delta. The result of this *in vitro* experiment inferred that there is increase in fusogenicity of BQ.1.1 and XBB.1, when compared with BA.1 and BA.2.

Cite this as: Sutandhio, S., Wihanto, L., Tedyanto, C.P., and Santoso, S. (2025). *In Vitro* Analysis of SARS-CoV-2 Variants that Caused Severe COVID-19 in the Elderly. *Indonesian Journal of Tropical and Infectious Disease*, 13(1) : 10–16. https://doi.org/10.20473/ijtid.v13i1.65484

INTRODUCTION

The etiological agent responsible for the coronavirus disease 2019 (COVID-19), the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has been widely known to have several variants. The original variant of SARS-CoV-2 that caused COVID-19 has been replaced by the S D614G variant, which was characterized by mutation in the gene encoding the S protein. The World Health Organization (WHO) reported five SARS-CoV-2 main variants that have distinctive differences compared to the original variant of SARS-CoV-2, including Alpha, Beta, Gamma, Delta, and Omicron.[1,2]

Omicron, the latest SARS-CoV-2 variant, caused milder symptoms compared to other previously detected variants. Omicron was also reported to have multiple mutations in the gene encoding the S protein, which increases the risk of SARS-CoV-2 reinfection after COVID-19 vaccination.[2]

Furthermore, Omicron has been observed to differentiate into several well-known variants, including BA.1, BA.2, BA.5, BQ.1.1, and XBB.1.[2] These variants have contributed to the abundance of COVID-19 cases worldwide. The abundant COVID-19 cases caused by Omicron did not escalate the concern because it has mild impacts on adults, but severe impacts leading to the risk of death usually occur in the elderly.[3,4]

Elderly is defined as people aged 65 years and above. Its population accounted for 28.9% of the population in 2021, according to the data from the Japanese government.[5] The immune response in the elderly gradually declines with time[4]. A previous study reported that the antibodies in elderly who had received four doses of the mRNA vaccine were still unable to optimally neutralize the BQ.1.1 and XBB.1 variants.[6]

We aim to investigate whether the increased severity of COVID-19 in the elderly was caused by the decrease of immunity or increase of virus pathogenicity. Here, we report an analysis of SARS-CoV-2 variants in cell cultures, including the BA.1, BA.2, BA.5, BA.2.75, BQ.1.1, XBB.1, S D614G, and Delta variants.

MATERIALS AND METHODS

This research was operated according to biosafety level 3 (BSL-3) protocols. Vero E6-TMPRSS2 cell was used because this cell line expresses the angiotensin-converting enzyme 2 (ACE2) receptor and the transmembrane protease serine 2 (TMPRSS2), which could help the virus to enter the cell. In addition, the nature of these cells that adhere to the bottom of the well and are low in interferon production serve as advantages in this research, in which SARS-CoV-2 is a virus that is sensitive to interferons.[7]

SARS-CoV-2 variants were inoculated into Vero E6-TMPRSS2 cell cultures to compare the diameter of the plaques formed. Eight variants were used, i.e., Omicron BA.1, Omicron BA.2, Omicron BA.5, Omicron BA.2.75, Omicron BQ.1.1, Omicron XBB.1, S D614G, and Delta. The source and whole genome sequencing identification of variants had been documented in the Global Initiative on Sharing All Influenza Data (GISAID) database under identification number EPI_ISL_7418017, EPI_ISL_9595859, EPI_ISL_13241867, EPI_ISL_13969765, EPI_ISL_15579783, EPI_ISL_15669344, LC644163 (DNA Data Bank of Japan), and EPI_ISL_2158617, respectively. These viruses were subcultured from isolates from

COVID-19 patients with low passage numbers.

The infected cells were incubated for seven days in a medium containing methylcellulose. After incubation, cells were rinsed, fixed, and stained using the crystal violet dye.

Plaque is an empty and clear area (unstained) seen as a transparent dot. It is formed as the dead cells detach from the base due to the cytotoxic effects of the virus (Figure 1). All experiments were performed in duplicate to obtain consistent results.

Single plaques are considered as the result from infection by a single virus. The width of the plaque depends on the ability of the virus to disseminate from the first infected cell to nearby cells. The plaques were analyzed using the ImageJ application to select single plaques and determine the mean plaque size or diameter.

The plaque sizes were entered as data for statistical analyses. The statistical analyses were performed using one-way analysis of variance (ANOVA), followed by pair-wise comparisons with independent samples t-test to compare differences in plaque size between each group. Data were analyzed using SPSS Statistics 25.0.

RESULTS AND DISCUSSION

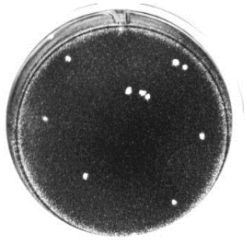

Figure 1. Crystal violet-stained plaque assay plate explains the cytopathic effect of the virus that formed clear plaques as transparent dots.

According to the analysis of 90 plaques from each variant, the mean plaque area were BA.1=BA.2<BA.5<BA.2.75<BQ.1.1<XB B.1. The plaque size of BA.1 were insignificantly different from the plaque size of BA.2. The plaque sizes of BQ.1.1 and XBB.1 were bigger that those of BA.1 and BA.2. However, the plaque area formed by several Omicron variants was still smaller when compared to S D614G and Delta variants. Table 1 shows the results of plaque size of SARS-CoV-2 variants on Vero E6-TMPRSS2 analyzed using ImageJ.

Table 1. Mean, median, and standard deviation of plaque size of SARS-CoV-2 variants on Vero E6-TMPRSS2

	Mean (mm^2)	Median (mm^2)	Standard Deviation
BA.1	0.106	0.107	0.035
BA.2	0.108	0.1	0.042
BA.5	0.163	0.16	0.026
BA.2.75	0.198	0.194	0.017
BQ.1.1	0.24	0.24	0.035
XBB.1	0.293	0.29	0.028
S D614G	0.308	0.305	0.03
Delta	0.384	0.388	0.053

One-way ANOVA statistical test revealed $p < 0.05$, indicating a difference between groups. The independent samples t-test showed that other than the plaque area of BA.1 and BA.2, there were significant differences between plaque area of the rest of the variants ($p < 0.05$). Figure 2 shows the plaque size comparisons of each SARS-CoV-2 variant, accompanied by the results of the two-tailed t-test analysis.

In a viscous media such as methylcellulose medium, SARS-CoV-2 infection relies on the fusogenicity of the virus, i.e., ability to form a membrane fusion between the infected and healthy cells. The fusion between cells causes the formation of syncytia, a multinuclear cell formed by multiple cell fusion.[8,9] The

formation of syncytia on S D614G and Delta variants could be observed two days after infection, while the syncytia on Omicron variants could be observed four days after infection. High fusogenicity and replication ability were found in the Delta variant of SARS-CoV-2, as revealed by the largest plaque size in this experiment. The association between the fusogenicity and SARS-CoV-2 pathogenicity remains unclear.

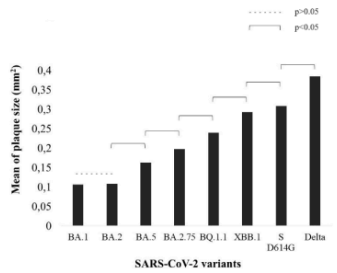

Figure 2. Plaque size comparisons of SARS-CoV-2 variants on Vero E6-TMPRSS2

It was initially thought that the fusogenicity of the virus was influenced by the P681 mutation in the gene encoding the S protein, a class I fusion glycoprotein that plays a role in the process of attachment and entry of the virus into cells. Moreover, it was discovered that the fusogenicity of the virus affects several factors, including the N-terminal domain and the cleavage process of the S protein, which results in the separation of the S1 and S2 regions in S protein.[10,11] Mutations in certain regions of the S protein can also cause changes in the fusogenicity of the virus.[10-15]

Omicron has undergone major mutations in its genes, including the S protein-encoding gene. In the early generation of Omicron, BA.1 and BA.2, there was a decrease in the use of TMPRSS2 during the process of virus entering the cells through the membrane fusion. Instead, the virus enters cells through a process called endocytosis. This theory explains why the fusogenicity of Omicron variants, BA.1 and BA.2, was low.[16,17] These findings also support the results of this research, which showed that BA.1 and BA.2 formed the smallest plaque area compared to other variants.

This research also revealed a trend in plaque size of the newer generation of Omicron variants (BA.5, BA.2.75, BQ.1.1, and XBB.1.), in which the plaque size tends to be larger than the old generation of Omicron variants. Only the BQ.1.1 and XBB.1 variants were observed to have almost the same plaque size compared to the S D614G variant. These findings show that SARS-CoV-2 is still evolving to find its ideal design.

STRENGTH AND LIMITATION

The strength of this study is that it is an *in vitro* study, where the variables can be limited to minimum. The use of live virus from different variants, instead of recombinants[10,15,18-21], could show the real virus behavior.

The use of Vero E6-TMPRSS2 cells could be a limitation in this research. Vero cells are a lineage of cells isolated from kidney epithelial cells from an African green monkey, not a human.[22-25]

CONCLUSIONS

In vitro results in this research revealed an increase in the fusogenicity of the BQ.1 and XBB.1 compared to the BA.1 and BA.2 variants. Further studies need to be conducted to confirm these results with clinical findings *in vivo*.

ACKNOWLEDGMENT

We thank Yasuko Mori, professor in Kobe University, for the laboratory use and virus isolates. We thank Youdiil Ophinni, assistant professor in Kyoto University, for the helpful advice in data analysis. We thank Yukiya Kurahashi, Lidya Handayani, Maria Istiqomah Marini, Gema Barlian Effendi, Khoir Amaliin, and Achmad Januar Er Putra, from Kobe University for the support throughout this experiment.

FUNDING

This study did not receive any funding.

ETHICAL CLEARANCE

This research protocol was approved by the Ethical Committee of Kobe University Graduate School of Medicine (approval no. B200200) on 15 April, 2022.

CONFLICT OF INTEREST

The authors do not have conflict of interest.

AUTHOR CONTRIBUTION

SSu designed the experiment, drafted the manuscript, and revised the manuscript. SSu and LW did the experiment. SSu and CPT did the data analysis. CPT translated the manuscript. SSa provided consultations.

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PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5

PAGE 6

PAGE 7