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The isolation rate of the severe acute respiratory syndrome coronavirus 2 Omicron variant strains using Vero lineage cells

Isao Yoshida, Mami Nagashima, Hiroyuki Asakura, Noeru Hasegawa, Takayuki Shinkai, Kenji Sadamasu

Department of Microbiology, Tokyo Metropolitan Institute of Public Health

Background: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) omicron variant has more mutations in "Spike" proteins than does SARS-CoV-2 delta variant and other variants and may be somewhat more difficult to isolate than SARS-CoV-2 delta variant, which is easily isolated in Vero E6/TMPRSS2 cells.
Objective: We investigated SARS-CoV-2 omicron variant strains in the maintenance medium (MM) with additives of various Vero lineage cells to determine whether or not they improve the isolation rates.
Methods: The MM and additives used after inoculation of Vero E6/TMPRSS2, VeroE6, and Vero cells in culture using SARS-CoV-2 omicron variant strains were examined. After the culture, the cellular denaturation effect (i.e., the cytopathic effect) (CPE) was observed on the cells, and superiority was determined from the dilution ratio of CPE-positive cells.
Results: High glucose Dulbecco's Modified Eagle's Medium was the best performing maintenance medium for the isolation and culture of SARS-CoV-2 omicron variant strains (BA.1 and BA.2). When Vero E6 cells were used for the isolation cultures, the addition of amphotericin B to the MM significantly improved the isolation rates.

Key words: SARS-CoV-2 omicron variant strains, Vero E6, Vero E6/TMPRSS2, amphotericin B, endocytic pathway

Introduction

In December 2019, an outbreak of pneumonia, later named coronavirus disease-19 (COVID-19), was reported in Wuhan, Hubei Province, People's Republic of China, and the causative virus was identified as a novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[1] Symptoms include fever, fatigue, breathlessness, abnormal taste and smell, and pneumonia, severe cases of which can be life-threatening. In Tokyo, the SARS-CoV-2 Wuhan variant, the Alpha variant, the Delta variant, and other mutant variants were detected and reported.[2] In addition, the Omicron (B.1.1.529) variant was first reported in South Africa in November 2021,[3] and the Omicron variant was reported in Tokyo from December and is now the prevalent variant, the mainstay of the disease.[4]

This Omicron variant is highly mutated compared with conventional variants, with increased angiotensin-converting enzyme 2 (ACE2) affinity (S:Q498R, N501Y), increased infectivity (S:H655Y, N679K, P681H), and other mutations in the "Spike" protein alone.[5] Moreover, whereas conventional variants bind to ACE2 as a receptor and utilize transmembrane serine protease 2 (TMPRSS2) when infecting cells,[6,7] the Omicron variant uses a TMPRSS2-independent dermal manner to efficiently enter cells via the endosomal route.[8,9]

Since January 2020, we have isolated and cultured SARS-CoV-2 using Vero E6 cells and Vero E6/TMPRSS2 cells[10,11] from clinical specimens (e.g., pharyngeal and nasal swabs and saliva) suspected of COVID-19. Regarding the Delta strain, it was relatively easy to isolate and culture SARS-CoV-2; however, with the Omicron variant, we encountered cases where isolation and culture was relatively difficult, regardless of the results of the real-time reverse transcript-

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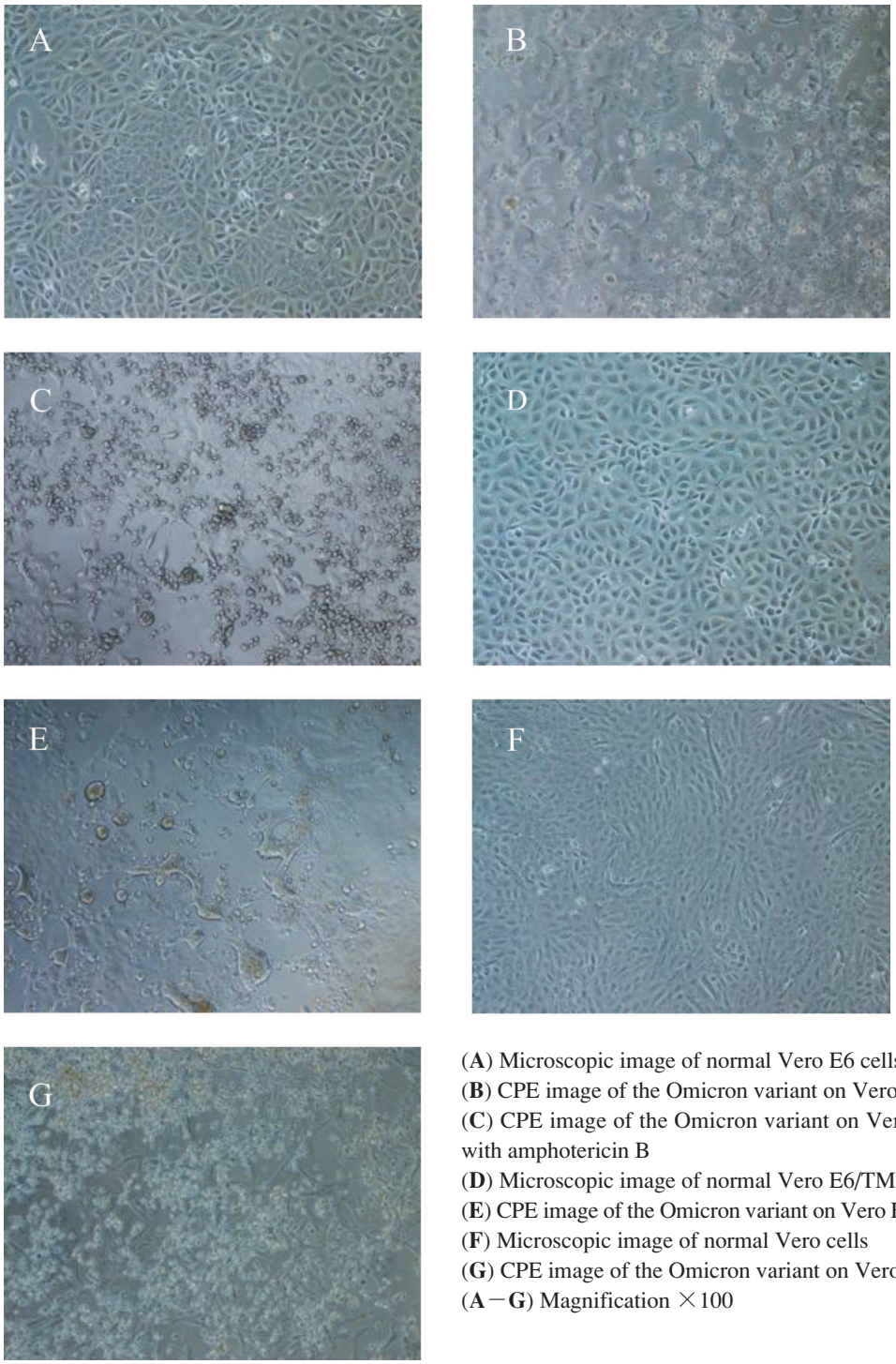
Correspondence to: Isao Yoshida, Department of Microbiology, Tokyo Metropolitan Institute of Public Health
3-24-1 Hyakunin-cho, Shinjuku, Tokyo 169-0073, Japan
E-mail: Isao_Yoshida@member.metro.tokyo.jp and isaoyo.6c6@gmail.com

polymerase chain reaction (RT-PCR) test (Cycle threshold [Ct] value), and other factors. Therefore, we herein report the findings of our investigation of culture conditions (e.g., media, concentrations, and temperature) for isolation cultures of the Omicron variant strains.

Materials and Methods

The maintenance medium (MM)

Omicron variant strains isolated by Vero E6 cells or Vero E6/TMPRSS2 cells from clinical specimens (pharyngeal and nasal swabs and saliva) suspected of having COVID-19 from public health centers in Tokyo were used. The Vero E6 (Distributed by Osaka Institute of Public Health) and Vero (CCCL-81, ATCC) cells used in the experiments



(**A**) Microscopic image of normal Vero E6 cells
(**B**) CPE image of the Omicron variant on Vero E6 cells
(**C**) CPE image of the Omicron variant on Vero E6 cells in MM with amphotericin B
(**D**) Microscopic image of normal Vero E6/TMPRSS2 cells
(**E**) CPE image of the Omicron variant on Vero E6/TMPRSS2 cells
(**F**) Microscopic image of normal Vero cells
(**G**) CPE image of the Omicron variant on Vero cells
(**A**－**G**) Magnification ×100

Figure 1. Inverted microscopic images of CPEs on the Omicron variant strains (S1539 and S2037) on different types of cells

were grown in Eagle-Minimum Essential Medium (E-MEM; Nissui Pharmaceutical, Tokyo) supplemented with 10% fetal bovine serum (FBS, Lot No. S15728S1530, Biowest, USA), 1% Non-Essential amino acids for MEM (NEAA; Nacalai Tesque, Kyoto), and 100 units/ml penicillin and 0.1 mg/ml streptomycin (Thermo Fisher Scientific, USA), as growth medium (GM); and Vero E6/TMPRSS2 (JCRB1819, JCRB Cell Bank) cells were grown in cultures in Dulbecco's Modified Eagle's Medium (D-MEM, Nissui Pharmaceutical) supplemented with 10% FBS, 1% NEAA, and 1 mg/ml G418 Bisulfate solution (Nacalai Tesque) and 100 units/ml penicillin and 0.1 mg/ml streptomycin as GM.

Omicron variant strains (S1539, Vero E6 2G: BA.1: Accession ID EPL_ISL_9847798 and S2037, Vero E6 2G: BA.2: Accession ID EPL_ISL_10117621) were diluted 10-fold in E-MEM with 2% FBS (10^{-1} to 10^{-5}). The diluted virus solution (100 μl/well) was inoculated into Vero E6, Vero E6/TMPRSS2, and Vero cells cultured in a monolayer in a 24-well cell culture plate (IWAKI, AGC Techno Glass, Tokyo) and allowed to adsorb in a 37℃ 5% CO_2 incubator for approximately 15 minutes. After adsorption, 2% FBS added E-MEM (FUJIFILM Wako Pure Chemicals Industries, Hamamatsu, Shizuoka) or 2% FBS added high glucose D-MEM-D7777 (Sigma-Aldrich, USA) was added as the maintenance medium (MM), and the cells were cultured in a 37℃ 5% CO_2 incubator for 7 days. The cytopathic effect (CPE) was observed under the MM (Nissui) using D-MEM (Nissui), not a high glucose formula, as the basic medium, and performed in the same way using Vero E6 and Vero E6/TMPRSS2 cells.

Examination of culture temperature

A 10-fold stepwise dilution (10^{-1} to 10^{-4}) of the Omicron variant strains in E-MEM was inoculated 50 μ/well into Vero E6 and Vero E6/TMPRSS2 cells cultured in monolayer on 24-well cell culture plates and incubated at 37℃ and 33℃. The MM was 2% FBS added enriched high glucose D-MEM (Sigma).

Influence of additives to the culture medium

We examined what would have the greatest impact on MM nutrients that would affect the results of the isolation and culture of the Omicron variant strains. The basic medium was D7777 (Sigma) as the standard medium, and glucose was added to the MM (Nissui) at 2 mg/ml, 4 mg/ml, or 0.11 mg/ml of sodium pyruvate (FUJIFILM Wako Pure Chemicals) to examine the effect of glucose on the development of the Omicron variant strains. The effect of CPE was determined by observing the expression of CPE under an inverted microscope.

Effect of amphotericin B concentration in the MM

Differences in the development of the Omicron variant strains of the MM (D-MEM; Sigma) supplemented with 3.125 μg/ml, 6.25 μg/ml, and 12.5 μg/ml amphotericin B (FUJIFILM Wako Pure Chemicals) and the MM without amphotericin B (D-MEM; Sigma) are shown in inverted microscope images comparing the CPEs (Figure 1).

Isolation and culture of the Omicron variant strains from clinical specimens

Seventy clinical specimens, collected from 70 persons suspected of having been infected with SARS-CoV-2, were brought in from public health centers in Tokyo. The 70 specimens included 45 cases (64.3%) of pharyngeal/nasal swab fluid, 21 cases (30.0%) of saliva, and 4 cases (5.7%) of unknown origin.

VeroE6 cells, Vero E6/TMPRSS2 cells, and Vero cells cultured in monolayer in 24-well cell culture plates, respectively, were inoculated with 100 μl/well of supernatant of the clinical specimens at 4℃ for 5 minutes at 9,500 rpm and centrifugation. In 25 cases where the volume of specimens was large, VeroE6 cells were inoculated by duplicate inoculation. After inoculation, adsorption was performed in a 37℃ 5% CO_2 incubator for at least 15 minutes. After adsorption, 1 ml/well of MM (Sigma) was added to each well of VeroE6, Vero E6/TMPRSS2, and Vero cells in the 24-well cell culture plate. Subsequently, 1 ml/well of the MM (Sigma) supplemented with 3,125 μg/ml amphotericin B was added to the other wells of Vero E6 cells, followed by incubation in a 37℃ 5% CO_2 incubator.

Next Generation Sequencing (NGS) analysis

The full-length sequence of the Omicron variant strains cultured in Vero E6 cells in MM with amphotericin B was determined by NGS and compared with the full-length sequence of the same virus, the Omicron variant strains cultured in MM without amphotericin B. RNA used for the library was extracted using the QIAmpViral RNA Mini Kit (QIAGEN, Tokyo), and NGS library preparation was performed using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB, Tokyo) and MiSeq (Illumina, Tokyo). Acquired data were continued using the ["Clinical"] CLC Genomics Workbench (CLC Bio, QIAGEN, Tokyo) and mapped to reference strains. Wuhan-Hu-1 (NC_045512.2) was used as a reference strain for mutation site searches.

Ethics

Approval from the Ethics Committee of the Tokyo Metropolitan Institute of Public Health and Safety Research Center (Approval No. 3KENKEN 465, May 31, 2021) was obtained to conduct this study.

Results

Examination of the medium

The results of the experiment comparing the development of the Omicron variant by D-MEM (Sigma), D-MEM (Nissui), and E-MEM showed that D-MEM (Sigma) was better than E-MEM for Vero E6 cells. In the MM study,

Table 1. Effect of maintenance medium on growth of Omicron variants strains (S1539 and S2037)

Cell type	Media	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Vero E6	D-MEM (Sigma)	+	+	+	+	−	−	−
	D-MEM (Nissui)	+	+	+	−	−	−	−
	E-MEM	+	+	+	−	−	−	−
Vero E6/ TMPRSS2	D-MEM (Sigma)	+	+	+	+	+	−	−
	D-MEM (Nissui)	+	+	+	+	−	−	−
	E-MEM	+	+	+	+	−	−	−

Table 2. Differences in development of Omicron variants strains (S1539 and S2037) as a function of incubation temperature

Temp.	Cell type	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
37℃	Vero E6	+	+	+	+	+	−	−
	TMPRSS2	+	+	+	+	+	+	+
	Vero	+	+	+	+	+	+	−
33℃	Vero E6	+	+	+	+	+	−	−
	TMPRSS2	+	+	+	+	+	+	−
	Vero	+	+	+	+	−	−	−

MM: D-MEM (Sigma)

Table 3. Effect of additives to the maintenance medium on the growth of Omicron variants strains (S1539 and S2037)

Cell Type	additive	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Vero E6	D-MEM (Sigma: Glucose concentration 4.5 mg/ml)	+	+	+	+	+	−
	No additives	+	+	+	+	−	−
	D-MEM Glucose, total 3 mg/ml	+	+	+	+	−	−
	(Nissui) Glucose, total 5 mg/ml	+	+	+	+	−	−
	Sodium pyruvate	+	+	+	+	+	−
	E-MEM	+	+	+	+	−	−
Vero E6/ TMPRSS2	D-MEM (Sigma:Glucose concentration4.5 mg/ml)	+	+	+	+	+	−
	No additives	+	+	+	+	−	−
	D-MEM Glucose, total 3 mg/ml	+	+	+	+	+	−
	(Nissui) Glucose, total 5 mg/ml	+	+	+	+	+	−
	Sodium pyruvate	+	+	+	+	+	−
	E-MEM	+	+	+	+	−	−

CPE appeared in Vero E6 cells with D-MEM (Sigma) at up to 10^{-4} dilution for both strains, S1539 and S2037. On Vero E6/TMPRSS2 cells, CPE (Figure 1) appeared at up to 10-5 dilution in D-MEM (Sigma), which was also 10-fold higher than that in E-MEM (Table 1).

Examination of culture temperature

In Vero E6 cells, there was no difference in the dilution factor at which CPE appeared between 33℃ and 37℃. However, in Vero E6/TMPRSS2 cells, the difference was 10-fold at 37℃; remarkably, in Vero cells, the difference was 100-fold at 37℃ (Table 2).

Effect of additives to the culture medium

In Vero E6 cells, addition of glucose (2 mg/ml and 4 mg/ml) to D-MEM (Nissui) did not significantly affect the dilution factor of CPE appearance. However, the addition of sodium pyruvate increased the dilution factor of CPE appearance to 10-fold, which was comparable to the control D-MEM (Sigma). On the other hand, in VeroE6/TMPRSS2 cells, both glucose and sodium pyruvate additions were better than E-MEM and comparable to D-MEM (Sigma) (Table 3).

Effects of amphotericin B concentration in the MM

In Vero E6 cells, CPE appeared up to 10^{-4} dilution in 0, 3.125, 6.25, and 12.5 μ/ml amphotericin B added to MM. In Vero E6/TMPRSS2 cells, CPE appeared up to 10^{-5} dilution with or without amphotericin B (Table 4).

Results of isolation of the Omicron variant strains from clinical specimens

The 70 clinical specimens tested included 45 from pharyngeal or nasal swabs, 21 from saliva, and 4 from unknown sources. Looking at the isolates by specimen type, 31 (77.5%) were isolated from pharyngeal or nasal swab fluid, 7 (17.5%) from saliva, and 2 (5.0%) from unknown sources (Table 5). By cells, 20 isolates (28.6%) were isolated with both Vero E6 and Vero E6/TMPRSS2 cells, 5 (7.1%) were isolated with Vero E6 cells only, and 15 (21.4%) were isolated with Vero E6/TMPRSS2 cells only (Table 6). The results of 25 isolates cultured with 3.125 μ/ml of amphotericin B added to the MM used for Vero E6 cells showed that 21 (84.0%) of the Omicron variant strains (BA.1 [18 strains]; BA.2 [3 strains]) were isolated (Table 7). Of the 21 strains that could be isolated from Vero E6 cells by amphotericin B with MM, 2 strains (BA.1, 2) could only be isolated by amphotericin B with MM.

Table 4. Effect of amphotericin B on growth of Omicron variants strains (S1539 and S2037)

Cell Type	Amphotericin B concentration	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Vero E6	0 μg/ml	+	+	+	−	−	−
	3.125 μg/ml	+	+	+	−	−	−
	6.25 μg/ml	+	+	+	−	−	−
	12.5 μg/ml	+	+	+	−	−	−
Vero E6/TMPRSS2	0 μg/ml	+	+	+	+	−	−
	3.125 μg/ml	+	+	+	+	−	−
	6.25 μg/ml	+	+	+	+	−	−
	12.5 μg/ml	+	+	+	+	−	−

Table 5. Breakdown of clinical samples used for isolation of Omicron variant strains and number of isolates.

Sample type	Swab fluid (pharyngeal or nasal)	Saliva	Unknown	Total
Sample	45	21	4	70
Isolations by sample	31	7	2	40
Isolation rate	77.5%	17.5%	5.0%	

Table 6. Results of isolation of Omicron variants strains from clinical samples

Samples	Vero E6 and Vero E6/TMPRSS2	Vero E6	Vero E6/TMPRSS2	Isolated	Non-isolated
70	20	5	15	40	30
Isolation rate	28.6%	71.1%	21.4%	57.1%	

Table 7. esults of isolation of the Omicron variant strains from clinical specimens by the MM with amphotericin B

Total number of samples	Vero E6 (+amphotericin B)	Vero E6 only	Vero E6/ TMPRSS2 only	Both Vero E6 and Vero E6/TMPRSS2	Total isolation
25	21 (84.0%)	1 (4.0%)	11 (44.0%)	7 (28.0%)	19 (76.0%)

(%): isolation rate

NGS analysis

Sequences in all regions of the Omicron variant strains cultured with 3.125, 6.25, and 12.5 μg/ml of amphotericin B added to the MM and in the Omicron variant strains cultured with the MM without amphotericin B were identical.

Discussion

A review of the literature on experiments dealing with the Omicron variant strains shows that Eagle-Minimum Essential Medium (E-MEM) is used in most of the media in culture experiments with the Omicron variant strains.[12,13] We, too, have been using E-MEM for the MM from the beginning of our isolation and culture of SARS-CoV-2. However, the Omicron variant strains did not necessarily give better isolation results than did the Delta variant; and we were able to improve the isolation rate by using high glucose D-MEM, which has a higher nutrient value than does that with E-MEM. In MM comparison experiments, a 10-fold difference in dilution factor was observed between Sigma's D-MEM and E-MEM for both Vero E6 and Vero E6/TMPRSS2 cells. However, for Vero E6 and Vero E6/TMPRSS2 cells, there was no significant difference in results between Nissui's D-MEM and E-MEM. This suggests that both cells are highly nutrient demanding in the culture of omicron mutant strains. Furthermore, in the case of Vero E6 cells, nutrient requirement may be higher if the virus takes a pathway that allows it to enter the cell by endocytosis after binding to ACE2. However, these details could not be proven in this experiment alone.

Omicron variant strains multiply the virus near the pharynx and have been reported to cause pharyngitis as a clinical manifestation, with fewer cases leading to pneumonia than those from the Delta variant strains.[8] Because upper respiratory tract infections are relatively common, we examined whether the omicron variant could growth at an incubation temperature of around 33℃, similar to that of rhinovirus.[14] The results showed that isolation was possible at 37℃, the conventional isolation and incubation temperature.

When the additives to MM were examined, the addition of sodium pyruvate to D-MEM (Nissui) increased the dilution factor at which CPE appeared in Vero E6 cells. However, the addition of glucose only (2 mg/ml and 4 mg/ml) had no effect on the dilution factor at which CPE appeared. This suggests that not only glucose but also sodium pyruvate may be the main cause of the increased isolation rate.

Omicron variant strains proliferate in a wide range of cells in the pharyngeal region because they proliferate efficiently by endocytosis without TMPRSS2 after binding to ACE2 and entering the cells.[8] At that time, there are interferon-inducible transmembrane proteins (IFITMs) that inhibit viral growth on membranes such as endosomes.[8,15,16] Amphotericin B inhibits the viral growth suppression of those IFITMs.8,15 When amphotericin B was added to the MM of the Omicron variant strains and their development examined, no significant differences were observed in either Vero E6 or Vero E6/TMPRSS2 cells in experiments using isolates.

The results of these studies were applied to the isolations and cultures of SARS-CoV-2 (the Omicron variants) from actual clinical specimens, D-MEM (Sigma) was used for the MM, and 37℃ was considered the

appropriate condition for the isolations and cultures.

The isolation rate of the Omicron variant was 40/70 (57.1%) by this method, while it was as high as 21/25 (84.0%) in Vero E6 cells with amphotericin B added to the MM, suggesting that the endocytic pathway was activated by the addition of amphotericin B. Furthermore, NGS gene analysis confirmed that the gene sequence of the omicron variant strain isolated in MM with amphotericin B matched the sequence of the virus isolated in MM without amphotericin B in all regions. Thus, it was confirmed that the addition of amphotericin B to MM did not affect the genes of the isolated omicron variant strains.

The Omicron variant strains can be easily isolated and cultured using Vero E6 cells and the MM with amphotericin B. We will continue to investigate whether or not the same effect can be obtained with other ACE2-bearing cells in future studes.

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Conflicts of Interest: None

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