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RESEARCH ARTICLE

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Virucidal effect of monogalactosyl diacylglyceride from a green microalga, *Coccomyxa* sp. KJ, against clinical isolates of SARS-CoV-2 as assessed by a plaque assay

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Abstract

Background: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of coronavirus disease 2019 (COVID-19) and is capable of human-to-human transmission and rapid global spread. Thus, the establishment of high-quality viral detection and quantification methods, and the development of anti-SARS-CoV-2 agents are critical.

Methods: Here, we present the rapid detection of infectious SARS-CoV-2 particles using a plaque assay with 0.5% agarose-ME (Medium Electroosmosis) as an overlay medium.

Results: The plaques were capable of detecting the virus within 36–40 h post-infection. In addition, we showed that a monogalactosyl diacylglyceride isolated from a microalga (*Coccomyxa* sp. KJ) could inactivate the clinical isolates of SARS-CoV-2 in a time- and concentration-dependent manner.

Conclusions: These results would allow rapid quantification of the infectious virus titers and help develop more potent virucidal agents against SARS-CoV-2.

KEYWORDS
detection time, infectious virus, natural product, quantification, virucidal action

1 | INTRODUCTION

Due to a new emerging RNA virus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2),[1] social and economic activities worldwide have come to a complete standstill. The virus has been identified as a coronavirus that is closely related to the 2003 SARS-CoV.[2] The rapid spread of SARS-CoV-2 has taken a lot of scientific effort for developing diagnostic, antiviral, and vaccine countermeasures. Therefore, there is an urgent need for the establishment of a high-quality viral detection and quantification method and the development of anti-SARS-CoV-2 agents.

SARS-CoV-2 is a single-stranded positive-sense RNA virus whose genome encodes four structural proteins, the nucleocapsid protein N that is a component of the capsid, the membrane protein and the envelope protein that are involved in the virus budding process, and the spike glycoprotein that is a key player in binding cell receptor and membrane fusion and virus entry into host cells.[3] In general, RNA viruses have higher mutation rates than DNA viruses,[4–6] because most

RNA viruses have single-stranded RNA and weak ability to repair gene mutations, while most DNA viruses have double-stranded DNA and high ability to repair the mutations. These mutations may sometimes weaken the efficacy of vaccines and/or antiviral agents that inhibit viral proliferation. That is, the purpose of the vaccine was production of the antibody against the viral proteins that bind to cells. When mutations occur in the viral proteins, however, the antibody might lose its binding ability to the mutant viruses, resulting in weakened vaccine efficacy. In comparison, virucidal agents that destroy viruses might be less susceptible to the mutations in viral RNA. Thus, an ideal antiviral drug should exhibit broad-spectrum activity against many variants, for example, by destroying the structure of envelope and/or capsid.

The virucidal activity of a sample is evaluated by various virus quantification assays. So far, SARS-CoV-2 titers have been reported by detecting viral RNA.[7,8] However, this method cannot specifically quantitate infectious virus particles. Infectious SARS-CoV-2 can be measured by 50% tissue culture infectious dose ($TCID_{50}$), which depends on detecting the presence or absence of the cytopathic effect caused by viral replication in cell cultures.[9] However, this method only provides a qualitative estimate of the infectious virus in $TCID_{50}$ units. Conversely, the plaque assay is a quantitative method of measuring infectious viruses by counting the plaque numbers formed in cell cultures upon infection with serial dilutions of a virus specimen. Therefore, the plaque assay remains the gold standard in determining the numbers of infectious virions.[10]

Monogalactosyl diacylglyceride (MGDG) is one of glycoglycerolipids contained in such as vegetables, fruits, and grains.[11] MGDGs from algae have been also reported as the components with anti-tumor and anti-inflammatory activities.[12,13] Previously, we reported the virucidal activity of MGDG obtained from a microalga, *Coccomyxa* sp. KJ, against herpes simplex virus type 2 (HSV-2),[14] an enveloped virus that causes genital herpes.[15,16] Physical changes in HSV-2 shape including a decrease in virus particle size and possible damage to the viral envelope were observed after MGDG treatment, as assessed using electron microscopy. In accordance with possible damage to the envelope, MGDG-treated HSV-2 showed no pathogenicity in an animal model. As SARS-CoV-2 is also enveloped virus, MGDG might cause any damage to the envelope, resulting in the loss of binding ability to host cells.

In this study, we describe the rapid quantification of infectious SARS-CoV-2 using a plaque assay and the evaluation of virucidal activity of MGDG against the virus by plaque titration. Additionally, we established conditions for the determination of plaque number within two days post-infection and also confirmed the inactivation of the clinical isolates of SARS-CoV-2 by MGDG.

2 | MATERIALS AND METHODS

2.1 | Chemicals

MGDG was obtained as an anti-HSV component from *Coccomyxa sp.* KJ, and its chemical structure has been determined to consist of α-linolenic acid (C18:3) (approximately 72%) and 7, 10, 13-hexadecatrienoic acid (C16:3) (approximately 23%) of the total fatty acids as reported previously.[14]

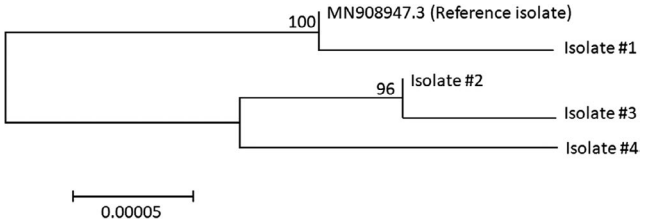

FIGURE 1 Dendrogram of SARS-CoV-2 strains obtained from clinical specimens

2.2 | Cell and viruses

The VeroE6/TMPRSS2 cell line, expressing transmembrane protease serine 2, was used in the present study. This cell line is highly susceptible to SARS-CoV-2 infection[17] and has been previously used for virus propagation and antiviral assays. The cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 μ/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 1 mg/ml geneticin, following removal of mycoplasma contamination by 50 μg/ml Plasmocure™ (InvivoGen). For antiviral assays, the cells were cultured in the absence of geneticin.

Four clinical isolates of SARS-CoV-2 obtained from Tokai University were used for the study. The SARS2-CoV-2 sequence from the four clinical isolates was compared with the sequence of reference strain Wuhan-Hu-1 (GenBank ID: MN908947.3). Whole RNA sequence analysis results of the clinical isolates are shown via a dendrogram in Figure 1.

2.3 | Plaque assay

The plaque assay was performed on VeroE6/TMPRSS2 cells. The cells (1×10^6 cells/dish) were grown in 35-mm dishes and infected with 10-fold serial dilutions of the virus at room temperature (26°C) for 1 h. Subsequently, 2 ml of an overlay medium was added. The overlay medium contained 1% SeaPlaque™ agarose (Lonza, Rockland, ME, USA), 0.8%, 1.2%, or 2% methylcellulose (MC) (4000cP, Wako Pure Chemical Industries, Ltd.), or 0.5% agarose-ME (Medium Electroosmosis) (classic type, Nacalai Tesque, Inc., Kyoto, Japan) in 1% FBS-DMEM. At 1, 2, or 3 days post-infection, the cells were fixed with 10% formaldehyde in phosphate-buffered saline (PBS) for 2 h at room temperature. The overlay medium was then removed, and the cell monolayers were stained with 0.06% crystal violet solution (20% ethanol in distilled water). The staining solution was removed to reveal the plaques.

2.4 | Virucidal assays

Virucidal assays have been used to determine whether a compound inactivates free viral particles outside of cells. The assay is performed

by incubating the virus with the test compound for a specified time followed by the determination of the remaining infectious viral particles.

In the present study, MGDG was tested at three concentrations of 10, 100, and 1000 μg/ml against four isolates of SARS-CoV-2 [1×10^5 plaque-forming units (PFU)/ml]. The remaining virus infectivity was calculated using a plaque assay as follows: MGDG and the virus samples were mixed and incubated at room temperature for 10, 30, 60, 180, and 360 min. PBS was tested in parallel as the negative control. Following the incubation period, the solutions were diluted 100-fold with PBS and each dilution was added to the cell monolayer in 35-mm dishes at 100 μl/dish. The cells were incubated at room temperature for 1 h, overlaid with DMEM containing 0.5% agarose-ME, and further incubated at 37°C in a 5% CO_2 incubator. After 36–40 h incubation, the dishes were observed for plaques.

3 | RESULTS

3.1 | Conditions for plaque formation in SARS-CoV-2-infected cells

Several overlay media were evaluated for plaque formation in virus-infected cells. Small plaques were formed after incubation for two to three days with 0.8% MC-DMEM overlay medium, while smaller plaques were formed with 1.2%–2% MC-DMEM medium. These plaques did not form a clean circle. Large plaques were formed after incubation for two days with 1% SeaPlaque-DMEM overlay medium; however, the rate of plaque formation was so fast that it was sometimes difficult to determine the appropriate fixation time. However, clear circular plaques of appropriate size appeared after 36–40 h post-infection in the cell cultures incubated with 0.5% agarose-ME-DMEM overlay medium.

Therefore, 0.5% agarose-ME-DMEM overlay medium was used in the subsequent virucidal assays.

3.2 | Isolation and RNA sequencing of clinical isolates of SARS-CoV-2

Whole RNA sequences of the clinical isolates were compared with the sequence of reference strain Wuhan-Hu-1. The sequence of clinical isolate #1 was much similar to the sequence of reference strain than those of clinical isolates #2, #3, and #4 (Figure 1). Interestingly, clinical isolates #2, #3, and #4 have a mutation D614G in the spike protein became dominant early in the COVID-19 pandemic and this mutation increases entry efficiency with enhanced ACE2-binding affinity.[18]

3.3 | Evaluation of virucidal effects of MGDG against SARS-CoV-2 by plaque assay

We assessed the virucidal effect of MGDG on SARS-CoV-2. The assay was based on incubating the MGDG-virus mixture prior to titration of the remaining virus infectivity via a plaque assay.

Figure 2 shows the plaque morphology of the clinical isolate #1 on VeroE6/TMPRSS2 cells at 40 h post-infection, where the virus was incubated in the absence (0 μg/ml MGDG) and in the presence of 10, 100, and 1000 μg/ml MGDG for 0 to 360 min.

As shown in Figure 3, the residual virus infectivity was reduced to approximately 50% after 30-min incubation and to less than 10% after 3 h incubation in the presence of 100 or 1000 μg/ml MGDG. No marked differences in virucidal activity of MGDG were observed among the four clinical isolates of SARS-CoV-2.

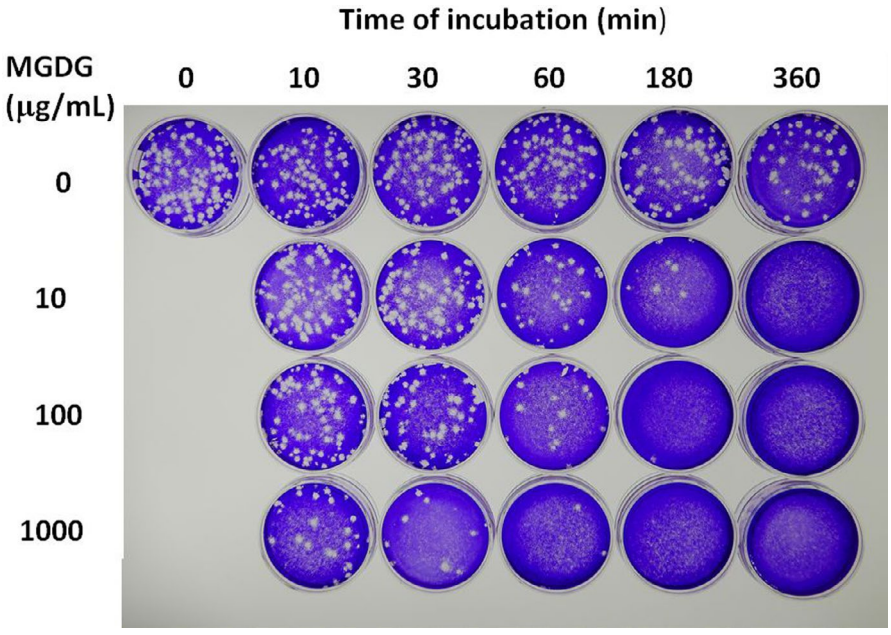

FIGURE 2 Plaque morphology of clinical isolate #1 of SARS-CoV-2 on VeroE6/TMRSS2 cells at 40 h post-infection using 0.5% agarose-ME-containing MEM as an overlay medium. The virus was treated with MGDG at concentrations of 0, 10, 100 and 1000 μg/ml for 0, 10, 30, 60, 180, and 360 min

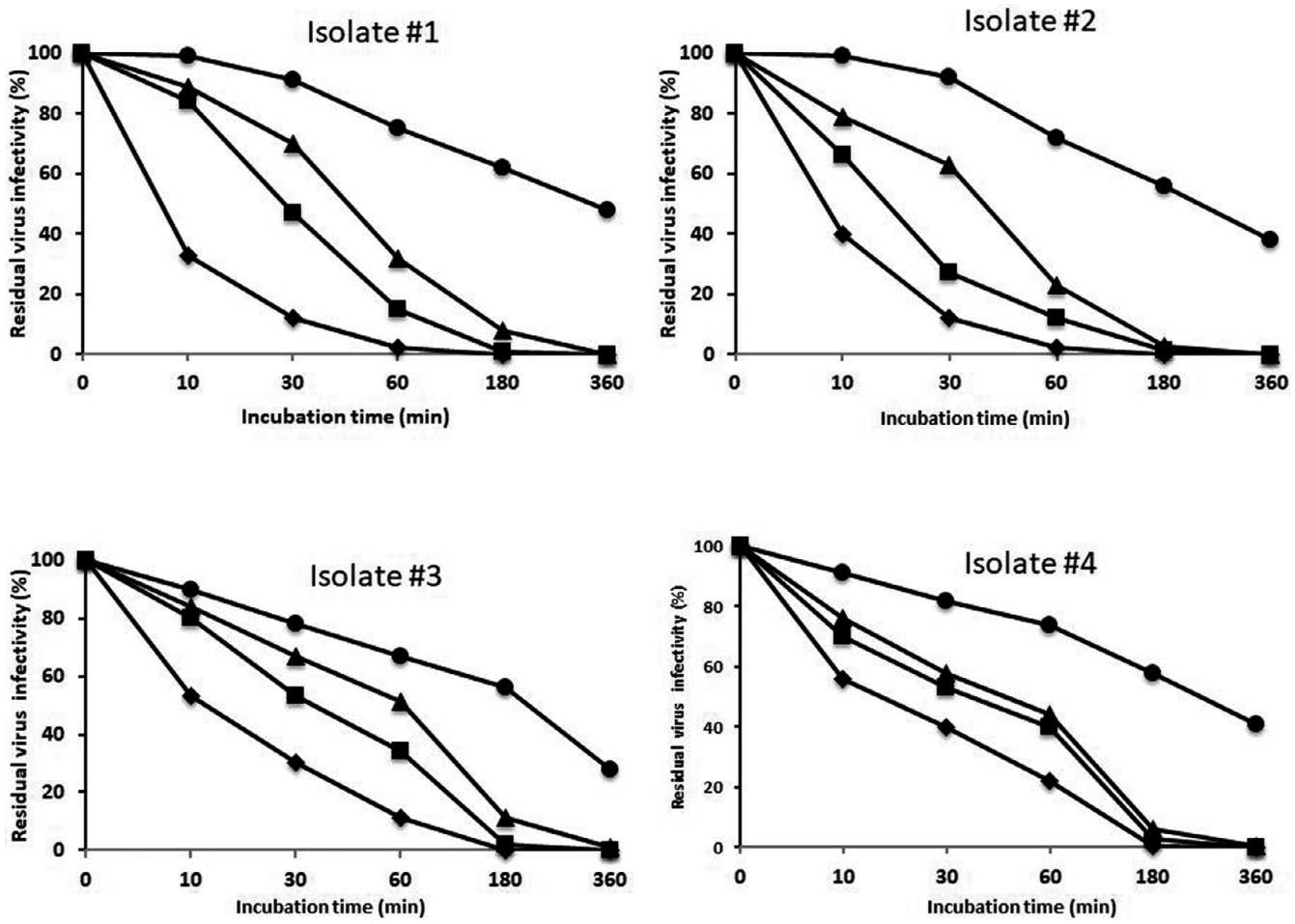

FIGURE 3 Virucidal activity of MGDG against four isolates of SARS-CoV-2. The virus sample (2×10^5 PFU/ml) was mixed with an equal volume of MGDG at specific concentrations (circle: 0 μg/ml, triangle: 10 μg/ml, square: 100 μg/ml, and rhombus: 1000 μg/ml) and incubated at 37°C for the indicated time. Results are expressed as the percentage of residual infectivity of MGDG-treated virus compared with the percentage of residual infectivity of the mock-treated virus control. Data are the means from independent duplicate assays

4 | DISCUSSION

There are many reports on the methods used to measure infectious SARS-CoV-2 particles by plaque assays with 0.375%–1% agarose, 0.6% or 1.2% microcrystalline cellulose, 1% MC, 2% Noble agar, 3% carboxymethylcellulose, or 0.8% Avicel RC-581 in the overlay medium (Table 1).[19–27] In these plaque assay conditions, two to four days are required to detect the plaques. On the contrary, in this study, we have described an assay that allows faster quantification of SARS-CoV-2 by detecting plaques formed in the cell monolayers within 36–40 h post-infection using 0.5% agarose-ME overlay medium.

There was no marked difference in the *in vitro* susceptibility of the four clinical isolates of SARS-CoV-2 to MGDG, regardless of mutation in the spike protein that affects binding activity to ACE2 (Figure 3). It is important to elucidate the virucidal mechanism of MGDG against SARS-CoV-2. As shown in a previous report,[14] MGDG caused a morphological change in the shape of HSV particles, possibly by damaging the viral envelope. HSV-2 and SARS-CoV-2 are

TABLE 1 Experimental conditions of plaque assay for SARS-CoV-2

Polymer present in the overlay medium	Time required for plaque detection	Reference
1% agarose	2 days	[19]
1% agarose	3 days	[20]
1% agarose	Not specified	[21]
0.4% agarose	3 days	[22]
0.6% or 1.2% microcrystalline cellulose	3 days	[22]
0.375% low-melting point agarose	3 days	[23]
1% methylcellulose	3 days	[24]
1% methylcellulose	4 days	[25]
2% Noble agar	3 days	[26]
3% carboxymethylcellulose	3 days	[26]
0.8% Avicel RC-581	3 days	[27]
0.5% Agarose-ME	1.5 days (36–40 h)	Present study

similar in that they have the envelope on the outermost side of the viral particle. While it is still unclear whether the damage to the viral envelope by MGDG happens in SARS-CoV-2 as well, these morphological changes might also contribute to the inactivation of all mutants of SARS-CoV-2 that may emerge in the future.

In conclusion, we have developed a rapid method for quantifying SARS-CoV-2 using a plaque assay. Additionally, we have described the inactivation of the virus by MGDG isolated from a microalga, *Coccomyxa* sp. KJ, using agarose-ME overlay medium.

In summary, we expect that the method of the plaque assay reported here will help determine the neutralizing antibody titers in serum samples such as those of COVID-19 patients, where the loss of viral infectivity by serum can be determined using a plaque assay. We also expect that MGDG could effectively act as a novel anti-SARS-CoV-2 agent through direct (virucidal) pathways.

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CONFLICT OF INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

K.H. designed and performed the experiments and wrote the article. S.A., K.U., H.K., and H.M provided the clinical specimens and analyzed them. M.M. and T.A. analyzed the RNA sequences of the isolated viruses. H.K. and S.K. isolated and analyzed the test sample. T.W. wrote the article. T.K. supervised the experiments. All of the authors have read and approved the final article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, K.H., upon reasonable request.

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