Comparison of Plaque Size, Thermal Stability, and Replication Rate among SARS-CoV-2 Variants of Concern

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18 **ABSTRACT**

19 SARS-CoV-2, like other RNA viruses, has a propensity for genetic evolution owing to the low fidelity
 20 of its viral polymerase. This evolution results in the emergence of novel variants with different
 21 characteristics than their ancestral strain. Several recent reports have described a series of novel SARS-
 22 CoV-2 variants. Some of these have been identified as variants of concern (VOCs), including alpha
 23 (B.1.1.7, Clade GRY), beta (B.1.351, Clade GH), gamma (P.1, Clade GR), and delta (B.1.617.2, Clade
 24 G). VOCs are likely to have some effect on transmissibility, antibody evasion, and changes in
 25 therapeutic or vaccine effectiveness. However, the physiological and virological understanding of
 26 these variants remains poor. We demonstrated that these four VOCs exhibited differences in plaque
 27 size, thermal stability at physiological temperature, and replication rates. The mean plaque size of beta
 28 was the largest, followed by those of gamma, delta, and alpha. Thermal stability, evaluated by
 29 measuring infectivity and half-life after prolonged incubation at physiological temperature, was
 30 correlated with plaque size in all variants except alpha. However, despite its relatively high thermal
 31 stability, alpha's small plaque size resulted in lower replication rates and fewer progeny viruses. Our
 32 findings may inform further virological studies of SARS-CoV-2 variant characteristics, VOCs, and
 33 variants of interest. These studies are important for the effective management of the COVID-19
 34 pandemic.

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38 **IMPORTANCE**

39 The global pandemic caused by SARS-CoV-2 continues to persist, due in part to mutations that have
 40 resulted in the emergence of different variants. Many of these variants have become more virulent and
 41 infectious than their ancestral strain, resulting in an ever-increasing spread. However, our virological
 42 understanding of these variants remains poor. Here, we directly compared the plaque size, stability,
 43 and replication kinetics of four SARS-CoV-2 variants of concern following prolonged incubation at
 44 physiological temperatures. Our observations may help to characterize each variant in terms of their
 45 interactions with host factors and responses to environmental conditions. We also believe that our
 46 evaluations will improve understanding of the emergence of new variants and contribute to controlling
 47 their spread.

48

49 **OBSERVATION**

50 Several novel variants of SARS-CoV-2 have been identified since the COVID-19 pandemic began in
 51 December 2019. Although the expected mutation rate for SARS-CoV-2 is estimated to be
 52 approximately 2.4×10^{-3} per site per year (1), significantly more mutations and deletions across
 53 thousands of variants, all of which may alter their pathogenic potential, have already been reported (2).
 54 The World Health Organization identifies novel SARS-CoV-2 variants of concern (VOCs) based on
 55 their potential impact on public health, according to evidence of enhanced transmissibility, increased
 56 severity, reduced antibody neutralization arising from former infection or vaccination, detection
 57 evasion ability, or decreased treatment or vaccine efficacy (3).

58 There are currently only four circulating SARS-CoV-2 VOCs: alpha (B.1.1.7, clade GRY), beta
 59 (B.1.351, clade GH), gamma (P.1, clade GR), and delta (B.1.617.2, clade G). They share D614G
 60 mutation, conferring increased infectivity, likely due to changes affecting the receptor binding and
 61 fusion (4-7). N501Y mutation is also shared by alpha, beta, and gamma, increasing their receptor-
 62 binding affinity and subsequent cellular entry (8). However, the combination of mutations could result
 63 in greater conformational changes and distinctive modifications (9). For example, VOCs exhibit
 64 differential receptor-binding affinity. Alpha requires the most force to be detached from the receptor,
 65 followed by beta/gamma and delta (10).

66 Here, we noted that each of these variants presented with different plaque sizes (Fig. 1a). The mean
 67 plaque size of beta was largest, followed by those of gamma, delta, and alpha (Fig. 1b). While there
 68 are numerous determinants of plaque size, we hypothesized that changes in the receptor-binding
 69 affinity, thermal stability, and replication rate of these viruses were likely factors. Based on the
 70 previously reported receptor-binding affinity data described above, we examined the thermal stability
 71 and replication rate of each of these VOCs. We first assessed the thermal stability of these variants in
 72 culture media incubated at different temperatures over an eight-hour period. Their infectivity was then

73 measured using a focus-forming assay. Owing to the short incubation time following viral infection,
74 this assay allowed for more precise assessments of virion infectivity, as it is less affected by other
75 factors associated with viral replication.

76 Of the four VOCs, the beta variant was the most stable at 4, 24, and 37 °C. We then evaluated the
77 relative stability by measuring changes in their infectivity following prolonged incubation (2, 4, 8, 12,
78 and 24 h) in solutions at physiological temperatures using the focus-forming assay. As expected, beta
79 exhibited the highest thermal stability (Fig. 1d), with a half-life approximately twice that of gamma or
80 delta (Fig. 1e). These results indicated a correlation between thermal stability and plaque size in all
81 variants except alpha. Interestingly, despite the small plaque size of alpha, its half-life was relatively
82 long, suggesting an alternative mechanism.

83 Next, we examined the viral replication rates of these VOCs. Vero E6 cells were infected with VOCs
84 at the same multiplicity of infection (MOI: 0.1). Plaque forming assay and qRT-PCR were then used
85 to assess infectious viral particle numbers and viral RNA concentrations, respectively. Alpha had fewer
86 infectious viral particles than the other variants, although there were no significant differences in viral
87 RNA copy number (Fig. 1f, 1g). Moreover, the intracellular viral RNA concentrations of alpha and
88 gamma were significantly lower than those of the other VOCs (Fig. 1h). These results indicate that the
89 viral replication rate of alpha is likely low, contributing to its small plaque size.

90 It is important to analyze these VOCs in terms of both their clinical pathology and virology, as this
91 will help us better understand their increased virulence. For example, plaque size may be associated
92 with contagiousness or viral transmission. Here, we noted that most of our VOCs presented with
93 changes in plaque size. In addition, these changes were largely mirrored by changes in virion stability
94 at physiological temperatures and the concentration of infectious viral particles. Correlation analyses
95 suggested that there is a strong link between plaque size and thermal stability in these VOCs and that
96 the relatively large plaque size of beta may account for its increased thermal resistance. This increased

stability may contribute to its pathobiology and transmission, requiring further studies of the viral titer and case fatality rate in humans. Conversely, although alpha presented with increased thermal stability, this did not translate to increased plaque size. This reduced plaque size may be explained by the reduced number of infectious particles produced by alpha under these conditions. Further studies will be required to identify additional determinants of plaque size, including functional mutations, interactions with host factors, and environmental composition. In addition, variants of interest and other variants should also be investigated to effectively control their spread.

Cell lines and viruses. Vero E6 cells (ATCC, CCL-1586) were maintained in Eagle's minimal essential medium (EMEM; WELGENE) supplemented with 10% fetal bovine serum (FBS; Gibco) and 100 U/mL penicillin-streptomycin (P/S; Gibco). SARS-CoV-2 variants (NCCP number: 43381 (alpha), 43382 (beta), 43388 (gamma), and 43390 (delta)) were obtained from the National Culture Collection of Pathogens of South Korea (NCCP). All experiments involving infectious SARS-CoV-2 variants were performed in a biosafety level 3 (BSL-3) containment laboratory at the Korean Research Institute for Chemical Technology (KRICT).

Viral titer determination Plaque- and focus-forming assays were performed as previously reported with some modifications (11). In brief, for the plaque assay, the virus was serially diluted in EMEM supplemented with 2% FBS. The cell culture medium was removed from Vero E6 cells (1×10^5 per 24-well) one day prior to the assay, before the inoculum was transferred onto triplicate cell monolayers. These cells were incubated at 37 °C for 1 h, and the inoculum was discarded before the infected cells were overlaid with 1.8% carboxymethyl cellulose in MEM. Samples were incubated at 37 °C for 4 d before they were fixed and stained using 0.05% crystal violet in 1% formaldehyde. Plaque counts and size evaluations were completed using an ImmunoSpot analyzer (C.T.L).

For the focus-forming assay, Vero E6 cells (2×10^4 per 96-well) were infected with diluted inoculum for 1 h and then placed in fresh medium for an additional 8 h, after which they were washed and fixed.

Cells were then stained with the N-specific antibody (Cat# 40143-R001, Sino Biological) and secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (Cat# 170-6515, Bio-Rad). The signal was then developed using an insoluble TMB substrate (Promega), and the number of infected cells was counted using an ImmunoSpot analyzer. Intra- and extracellular viral RNA was quantified using quantitative RT-PCR (QuantStudio 3, Applied Biosystems), which was completed using the one-step Prime script III qRT-PCR mix (Takara). Viral RNA was detected using a 2019-nCoV-N1 probe (Cat#10006770, Integrated DNA Technologies).

Statistical analysis. Mean values for the plaque size and half-life assays were compared using one-way ANOVA in GraphPad Prism 8.0 software (GraphPad Software). Statistical significance was set at $p < 0.05$.

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The authors have no conflicts of interest to declare.

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FIGURE LEGENDS

FIG. 1 Comparative analyses of the plaque size, thermal stability, and replication rate of four SARS-CoV-2 variants. (A) Representative images of the plaque forming assay for each variant of concern. (B) The mean plaque size ($1\text{E-}3\text{ mm}^2$) of each variant. (C) The relative infectivity of each variant after incubation at 4, 24, or 37 °C for 8 h as evaluated via the focus-forming assay. (D) Nonlinear regression of the relative infectivity of each variant following prolonged incubation (2, 4, 8, 12, and 24 h) at physiological temperatures (37 °C). (E) Average half-life values for each variant at 37 °C. (F) The infectivity of the progeny viruses was evaluated via plaque forming assay completed at 12, 24, and 48 h post infection. (G) Extracellular and (H) intracellular viral RNA was assessed by qRT-PCR with SARS-CoV-2 NP probes. All error bars indicate the standard errors of the mean. Mean values for the plaque size and half-life were compared using one-way ANOVA in GraphPad Prism 8.0 software. Statistical significance was set at $p < 0.05$.

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