

## Differences in New Variant of Concern Replication at Physiological Temperatures In Vitro

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Using multiple cell types and isolates of Delta and Omicron variants of SARS-CoV-2, we report differences in virus production, replication, and infectivity in vitro. Ancestral and Delta SARS-CoV-2 variant exhibit reduced virus production and replication at 34°C compared to 37°C while Omicron replication is balanced between temperatures.

**Keywords.** SARS-CoV-2; COVID-19; new variant of concern; Omicron variant; replication; transmission; temperature sensitivity; Delta variant.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the etiologic agent of coronavirus disease 2019 (COVID-19), which has persisted for years at the cost of millions of lives and incalculable economic damage. Despite the presence of viral RNA proofreading, SARS-CoV-2 has proven unexpectedly agile, with a spike protein mutation rate exceeding the hemagglutinin evolution of pandemic influenza virus by 2.5 times [1]. Specific antigenic drift of the spike protein seems to associate with variant transmission and exceeds the nonsynonymous mutation rate of the RNA-dependent RNA polymerase. Spike mutations have been associated with increased infection [2], transmission [3], and evasion of neutralizing antibodies [4]. Despite being more transmissible, the SARS-CoV-2 Omicron variant was found to cause milder diseases in laboratory animals, often accompanied by a lower viral load compared to previous variants of concern. The emergence of SARS-CoV-2 Omicron variant (B.1.1.529) has drastically changed the landscape of the COVID-19 pandemic [5]. Omicron displaced the Delta variant due to increased transmissibility and possibly environmental stability [6], although replication and pathogenesis of Omicron appears to be diminished in cell culture, animal models, and patients compared to previous variants [7].

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## METHODS

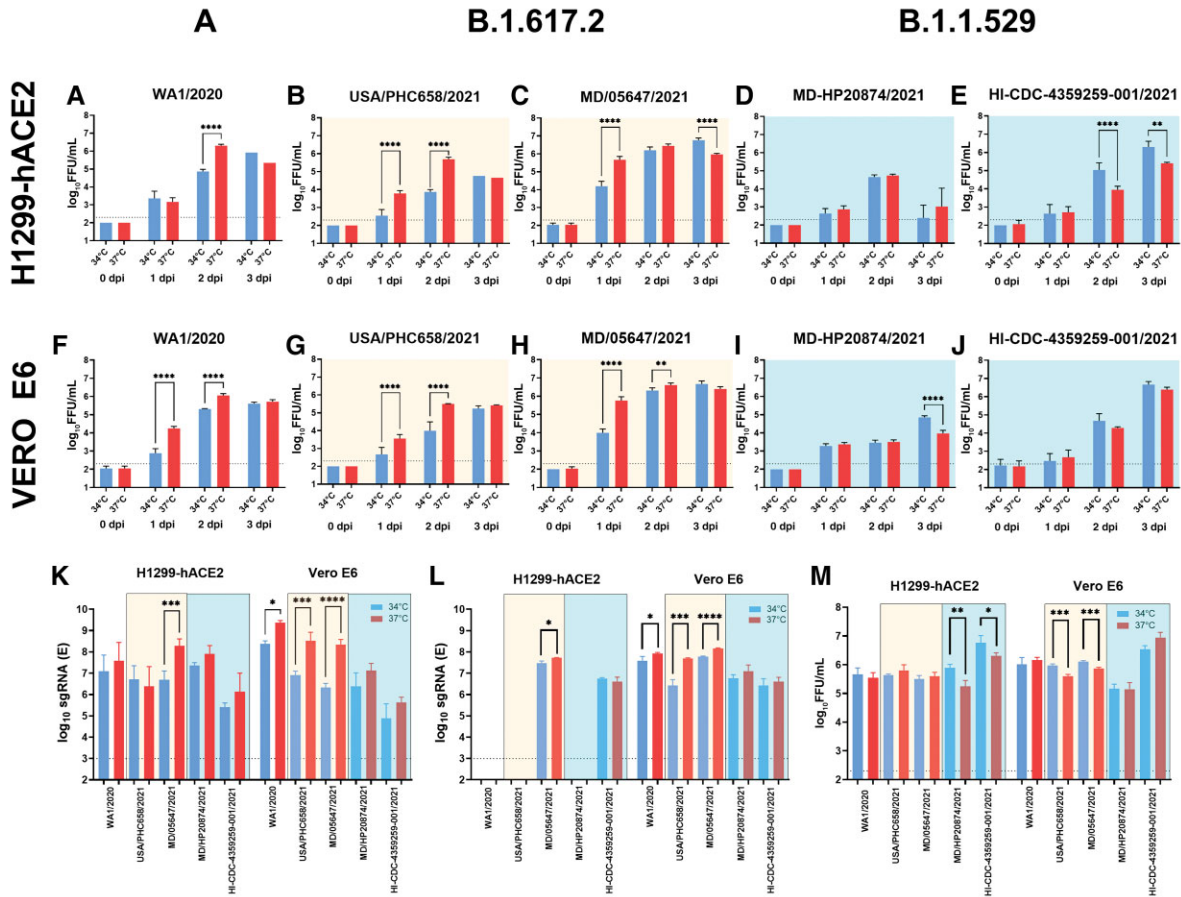
Vero E6 cells (catalogue No. CRL-1586) were purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen), and 1% penicillin/streptomycin and L-glutamine. H1299-hACE2 (NR-53821) cells were obtained from BEI Resources (National Institute of Allergy and Infectious Diseases, National Institutes of Health) and maintained in DMEM supplemented with 5% penicillin and streptomycin, and 10% FBS at 37 °C with 5% CO<sub>2</sub>.

SARS-CoV-2 isolate WA1/2020 (NR-52281, lot 70033175) was obtained from BEI Resources passaged 3 times in Vero cells prior to acquisition. It was further passed once in Vero E6 cells in our lab to generate viral stocks. SARS-CoV-2 Delta variants (Pango lineage B.1.617.2) hCoV-19/MD/05647/2021 (NR-55672) and hCoV-19/USA/PHC658/2021 (NR-55611) were obtained from BEI Resources. Delta variants were passaged once more in H1299-hACE2 cells in our laboratory to generate viral stocks. Omicron (Pango lineage B.1.1.529) hCoV-19/USA/MD-HP20874/2021 (NR-56462; MD-HP) and hCoV-19/USA/HI-CDC-4359259-001/2021 (NR-56475; HI-CDC) were obtained from BEI Resources and used directly in experiments. Passaged viruses were deep sequenced to confirm identity. Two isolates of Delta and two isolates of Omicron BA.1 were used to infect Vero E6 and H1299-hACE2 cells at a multiplicity of infection (MOI) of 0.001 in addition to WA1/2020 ancestral SARS-CoV-2. Multiplicities of infection were calculated using H1299-hACE2 focus-forming assay titers conducted at 37°C. Each growth curve was conducted with 9 replicates in separate wells of tissue-culture-treated 6-well plates (Corning).

Viral RNA was extracted from infected cell lysates using TRIzol reagent (Thermo Fisher) and QIAgen RNeasy kit followed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using primer/probes specific for the E region as described previously [8]. Focus-forming assays were conducted in H1299-hACE2 cells as described previously [4]. Growth curves were analyzed using mixed-effects analysis and viral RNA/focus-forming unit (FFU) data analyzed using multiple unpaired *t* tests using GraphPad Prism version 9.3.1 for Windows.

## RESULTS

Replication of Omicron and Delta isolates were compared to ancestral WA1/2020 in 2 cell lines at 2 physiological temperatures mimicking the conditions of the upper (34°C) and lower (37°C) respiratory tracts. Significantly lower viral titers were observed at multiple time points postinfection at 34°C for



**Figure 1.** Replication of ancestral and Delta variant but not Omicron variant SARS-CoV-2 is reduced at lower temperature *in vitro*. H1299-hACE2 cells (A–E) and Vero E6 cells (F–J) were infected at a multiplicity of infection of 0.001 and incubated at 34°C or 37°C for 3 dpi with WA1/2020 (ancestral SARS-CoV-2), B.1.617.2 (Delta), or B.1.1.529 (Omicron). Viral replication was assessed by measuring levels of subgenomic E RNA at 1 dpi (K) and 2 dpi (L) in infected H1299-hACE2 and Vero E6 cells at 34°C and 37°C. Specific infectivity was addressed by using the same viral stock to infect H1299-hACE2 or Vero E6 cells at 34°C or 37°C in focus-forming assays to compare viral titers (M). Tan shading indicates Delta isolates and blue shading indicates Omicron isolates. Abbreviations: A, ancestral; dpi, days postinfection; FFU, focus-forming unit; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; sgRNA, subgenomic RNA.

WA1/2020 (ancestral), USA/PHC658/2021 (Delta), and MD/05647/2021 (Delta) in both cell lines. Lower viral titers were more pronounced 1 and 2 dpi (days postinfection) in Vero E6 cells for all 3 viruses, with 1–2  $\log_{10}$  reduced FFU titers at 34°C. However, by 3 dpi viral titers were comparable at both temperatures for ancestral (Figure 1A and 1F) and Delta variant virus-infected cells (Figure 1B, 1C, 1G, and 1H). The recovery of viral titers at 34°C at 3 dpi was accompanied with a concomitant decline in titers at 37°C due to widespread cytopathic effect first observed at 2 dpi in H1299-hACE2 cells infected with WA1/2020 (Figure 1A) and USA/PHC658/2021 (Figure 1B). Conversely, viral titers were either similar or higher at 34°C for Omicron variants MD-HP and HI-CDC with significant approximately 1  $\log_{10}$  increases observed at 2 and 3 dpi in HI-CDC-infected H1299-hACE2 cells (Figure 1E) and 3 dpi MD-HP-infected Vero E6 cells (Figure 1I). Unlike WA1/2020 and the Delta isolates, no reduction in infectious virus titer

was observed in the supernatant of Omicron-infected cells (Figure 1A–1J). Instead, at 34°C significantly increased virus production was observed in H1299-hACE2 cells infected with HI-CDC at 2 dpi and 3 dpi (Figure 1E) as well as at 3 dpi in Vero E6 cells infected with MD-HP (Figure 1I).

To determine the underlying mechanism behind the reduced viral titers, subgenomic viral RNA of the E gene was used to measure viral replication in infected cells by qRT-PCR. A greater than 10-fold drop in subgenomic RNA copies was observed in MD/05647/2021-infected H1299-hACE2 cells at 1 dpi (Figure 1K). Reduced viral replication corresponded with less viral production in WA1/2020 and Delta variant-infected Vero cells at 1 dpi. Although less infectious virus was produced by USA/PHC658/2021 (Delta)-infected H1299-hACE2 cells (Figure 1B), comparable levels of subgenomic RNA were present at both temperatures (Figure 1K). Due to widespread cell death of WA1/2020, USA/PHC658/2021, and MD-HP infected

H1299-hACE2 cells at 2 dpi, RNA was not recovered for analysis from infected cells. However, viral RNA recovered from groups with intact monolayers showed significantly lower viral replication in WA1/2020 and Delta variant infected Vero E6 cells at 2 dpi (Figure 1L). Viral replication for each Omicron isolate was similar at 34°C and 37°C for infected H1299-hACE2 and Vero E6 cells.

SARS-CoV-2 spike binding to the ACE2 receptor is known to be temperature dependent [9], which could lead to differences in specific infectivity causing downstream effects on viral replication and production. To explore this possibility, viral stocks of WA1/2020, Delta, and Omicron SARS-CoV-2 were titrated in H1299-hACE2 and Vero E6 cells by focus-forming assay to measure the infectivity in each cell line at 34°C and 37°C for each isolate (Figure 1M). Infectivity of H1299-hACE2 cells by Omicron variants was significantly higher at 34°C compared to 37°C, with the same viral stocks infecting more cells at the lower temperature. In Delta variant-infected Vero E6 cells, the infectivity was also higher at 34°C compared to 37°C for each isolate.

## DISCUSSION

The (basic reproduction number)  $R_0$  value of the SARS-CoV-2 Omicron is reportedly 7.0 or greater, several times that of SARS-CoV-2 Delta [10], putting it among one of the most transmissible respiratory viral pathogens. Paradoxically, multiple studies reported seeming attenuation of Omicron in cultures, animal models, and humans [11, 12].

In this study, we characterized Omicron BA.1 strains in Vero E6 as well as human lung H1299-hACE2 cells at temperatures representing the upper and lower respiratory tract. Omicron isolates retained infectivity in these cell lines at both temperatures. By contrast, viral replication and production were significantly abrogated at lower temperatures for ancestral and Delta variants. Altogether, these findings imply a possible growth advantage of Omicron variant at lower temperature. Replication of Delta and ancestral SARS-CoV-2 were inhibited at 34°C, a temperature equivalent to that in the upper respiratory tract. Reduced viral production of Delta isolates and WA1/2020 seem to be due to loss of viral replication, supported by equivalent infectivity and reduced subgenomic viral RNA levels in infected cells at 34°C relative to 37°C. Both Omicron isolates examined in this study displayed a 2.5- to 4.0-fold increase in infectivity in a human lung cell line at 34°C compared to 37°C. Hence, Omicron appears to have adapted to infection at a temperature corresponding to the upper respiratory tract. Such a unique feature was not observed with the ancestral or 2 Delta isolates. However, it must be stated that Omicron replication at 34°C did not exceed that of ancestral or Delta variants. The effects of temperature on virus activity differed slightly between the laboratory workhorse Vero E6 cells and the human lung cell line

H1299-hACE2. Additionally, substantial variation was seen between the 2 Omicron isolates evaluated, with HI-CDC replicating to higher titers in both cell lines. Clinical studies seeking to determine infectious viral loads or isolate SARS-CoV-2 from patient samples should be cautious about the temperature settings used for in vitro cultures. SARS-CoV-2 studies conventionally use viral propagation and titration protocols at 37°C, which may exaggerate attenuation of Omicron isolates or undercount infectious particles. Additional studies using primary human nasal epithelial cells may help to understand the role of temperature in the increased transmission of emerging Omicron subvariants (BA.2, BA.3, and BA.4).

## Notes

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