Isolation and characterization of SARS-CoV-2 from the first US COVID-19 patient

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Article Summary: Scientists have isolated replication competent virus from the first US COVID-19 patient. The isolated virus described here will serve as the US reference strain to be used in research, drug discovery and vaccine testing.

Running title: Isolation of SARS-CoV-2 USA-WA1/2020

Keywords: Coronavirus, SARS-CoV-2, COVID-19, virus isolation

ABSTRACT

The etiologic agent of the outbreak of pneumonia in Wuhan China in January-2020, was identified as severe acute respiratory syndrome associated coronavirus 2 (SARS-CoV-2) . The first US patient was diagnosed by the State of Washington and the US Centers for Disease Control and Prevention on January 20, 2020. We isolated virus from nasopharyngeal and oropharyngeal specimens, and characterized the viral sequence, replication properties, and cell culture tropism. We found that the virus replicated to high titers in Vero-CCL81 cells and Vero E6 cells in the absence of trypsin. We also deposited the virus into two virus repositories, making it broadly available to the public health and research communities. We hope that open access to this important reagent will expedite development of medical countermeasures.

BACKGROUND

A novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has been identified as the source of a pneumonia outbreak in Wuhan China in late 2019 (1, 2). The virus was found to be a member of the beta coronavirus family, in the same species as SARS-CoV and SARS-related bat CoVs (3, 4). Patterns of spread indicate that SARS-CoV-2 can be transmitted person-to-person, and may be more transmissible than SARS-CoV (5-7). The spike protein, one of the structural outer proteins of coronaviruses, mediates virus binding and cell entry. Initial characterization of SARS-CoV-2 spike protein indicate that it binds the same receptor (ACE2) as SARS-CoV, which is expressed in both the upper and lower human respiratory tracts (8). The unprecedented rapidity of spread of this outbreak presents a critical need for reference reagents for further research on SARS-CoV-2. The public health community requires viral lysates to serve as diagnostic references, and the research community needs virus isolates to test anti-viral compounds, develop new vaccines, and perform basic research. In this

manuscript, we describe isolation of virus from the first US COVID-19 patient and described its genomic sequence and replication characteristics. We have made the virus isolate available to the public health community by depositing into two virus reagent repositories, BEI resources and The World Reference Center for Emergine Viruses and Arboviruses.

RESULTS and DISCUSSION

A patient with confirmed COVID-19 was identified in Washington State on January 22, 2020 with cycle threshold (C_ts) of 18-20 (nasopharyngeal (NP)) and 21-22 (oropharyngeal (OP)) (1). The positive clinical specimens were aliquoted and refrozen, then inoculated into cell culture on January 22, 2020. We first observed cytopathic effect (CPE) 2 days post inoculation and harvested viral lysate on day 3 post inoculation (Figure 1B and 1C). Fifty µl of first passage (P1) viral lysates were used for nucleic acid extraction to confirm the presence of SARS-CoV-2 using the CDC molecular diagnostic assay, which tests three regions of the nucleocapsid (N) gene by reverse transcription real time PCR (rRT-PCR) (1). The C_ts of three different rRT-PCRs ranged from 16.0-17.1 for N1, 15.9-17.1 for N2 and 16.2-17.3 for N3, confirming isolation of SARS-CoV-2. A C_t of less than 40 is considered positive (10). The extracts were also tested for the presence of 33 additional different viral and bacterial respiratory pathogens with the Fast Track 33 assay (FTD diagnostics, Silema, Malta). No other pathogens were detected. Identification was additionally supported by thin section electron microscopy (Figure 1D). We observed the morphology and morphogenesis characteristic of coronaviruses.

Isolates from the first passage of an OP and an NP specimen were used for whole genome sequencing. The genomes from the NP specimen (Genbank accession MT020880) and OP specimen (Genbank accession MT020881) matched each other 100%. The isolates also matched the corresponding OP clinical specimen 100% (Genbank accession MN985325).

After the second passage, OP and NP specimens were not cultured separately, and the isolate was named SARS-CoV-2 USA-WA1/2020. Virus isolate was passaged two more times in Vero CCL-81 cells, and titrated by TCID50. The titers of the third and fourth passages were 8.65 x 10^6 and 7.65 x 10^6 TCID50 per mL, respectively.

Of note, we passaged this virus in the absence of trypsin. The spike protein coding sequence of SARS-CoV-2 has an RRAR insertion at the S1-S2 interface that may be cleaved by furin (11). Highly pathogenic avian influenza viruses have highly basic furin cleavage sites at the hemagglutinin protein HA1-HA2 interface that permit intracellular maturation of virions and more efficient viral (12). The RRAR insertion in SARS-CoV-2 may serve a similar function.

We subsequently generated a fourth passage stock of SARS-CoV-2 on VeroE6 cells, another fetal rhesus monkey kidney cell line. Viral RNA from SARS-CoV-2 passage four stock was sequenced and confirmed to have no nucleotide mutations compared with the original reference sequence (Genbank accession MN985325). Both SARS-CoV and MERS-CoV had been found to grow well on VeroE6 and Vero CCL81 respectively (13, 14). To establish a plaque assay and determine the preferred Vero cell type for quantification, we titered our passage four stock on VeroE6 and VeroCCL81. Following infection with a dilution series, we found that SARS-CoV-2 replicated in both Vero cell types; however, the viral titers were slightly higher in VeroE6 cells than Vero CCL81 (**Figure 2A**). In addition, plaques were more distinct and visible on Vero E6 (**Figure 2B**). As early as 2 days post inoculations, VeroE6 cells produced distinct plaques visible with neutral red staining. In contrast, Vero CCL81 produced less clear plaques and was most easily quantitated with neutral red 3 days post inoculation. On the individual plaque monolayers, SARS-CoV-2 infection of Vero E6 cells produced cytopathic effect with areas of cell clearance (**Figure 2C**). In contrast, Vero CCL81 had areas of dead cells that had

fused to form plaques, but the cells did not clear. Together, the results suggest that VeroE6 may be the best choice for amplification and quantification, but both Vero cell types support amplification and replication of SARS-CoV-2.

As research is initiated to study and respond to SARS-CoV-2, information about cell line tropism is needed. Therefore, we examined the capacity of SARS-CoV-2 to infect and replicate in several common primate and human cell lines, including human adenocarcinoma cells (A549), human liver cells (HUH7.0), and human embryonic kidney cells (HEK-293T), in addition to Vero E6 and Vero CCL81. We also examined an available big brown bat kidney cell line (EFK3B) for SARS-CoV-2 replication capacity. Each cell line was inoculated with at high MOI and examined 24 hours post infection (**Figure 3A**). No cytopathic effect was observed in any of the cell lines except in Vero cells which grew to $>10^7$ PFU at 24 hours post infection. In contrast, both HUH7.0 and 293T cells showed only modest viral replication and A549 cells were incompatible with SARS-CoV-2 infection. These results are consistent with previous susceptibility findings for SARS-CoV(15). In addition, SARS-CoV-2 failed to replicate in the bat EFK3B cells which are susceptible to MERS-CoV. Together, the results indicate that SARS-CoV-2 maintain a similar profile to SARS-CoV in terms of susceptible cell lines.

Having established robust infection with SARS-CoV-2 in several cell types, we next evaluated the cross reactivity of SARS-CoV antibodies against the SARS-CoV-2. Cell lysates from infected cell lines were probed for protein analysis; we found that antibodies against the SARS-CoV spike and nucleocapsid proteins recognize SARS-CoV-2 (**Figure 3B & C**). The N protein, highly conserved across the group 2B family, retains >90% amino acid identity between SARS-CoV and SARS-CoV-2. Consistent with the replication results (**Figure 3A),** SARS-CoV-2 showed robust N protein in both Vero cell types, less protein in HUH7.0 and 293T, and

minimal signal in A549 and EFK3B cells (**Figure 3B**). Similarly, the SARS-CoV spike antibody also recognized SARS-CoV-2 spike protein, indicating cross reactivity (**Figure 3C**). Consistent with SARS CoV, several cleaved and uncleaved forms of the SARS-CoV-2 spike protein. Notably, the cleavage pattern to the the SARS spike positive control from Calu3 cells, a respiratory cell line, varies slightly and could signal differences between proteolytic cleavage of the spike proteins between the two viruses. However, differences in cell type and conditions complicate this interpretation and indicate the need to further study in equivalent systems. Overall, the protein expression data from SARS-CoV N and S antibodies recapitulate replication findings and indicate that SARS-CoV reagents can be utilized to characterize SARS-CoV-2 infection.

The SARS-CoV-2 USA-WA1/2020 viral strain described above has been deposited into BEI reagent resources (ATCC) and the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA, UTMB) to serve as the SARS-CoV-2 reference strain for the United States. The SARS-CoV-2 fourth passage virus has been sequenced and the nucleotide sequence is identical to that of the original US clinical strain. This deposit makes it available to the domestic and international public health, academic, and pharmaceutical sectors for basic research, diagnostic development, antiviral testing, and vaccine development. We hope broad access will expedite countermeasure development and testing, in addition to facilitating a better understanding of the transmissibility and pathogenesis of this novel emerging virus.

METHODS

Specimen collection

Virus isolation from patient samples was deemed to be non-human subjects research by CDC National Center for Immunizations and Respiratory Diseases (research determination 0900f3eb81ab4b6e). Clinical specimens from the first identified US case of COVID-19 acquired during travel to China, were collected as described (1). Nasopharyngeal (NP) and oropharyngeal (OP) swabs in 2 to 3 mL viral transport media were collected on day 3 post-symptom onset for molecular diagnosis (https://www.cdc.gov/coronavirus/2019-nCoV/lab/guidelines-clinical-specimens.html) , shipped on cold packs, extracted, used for molecular diagnostics, and frozen. Confirmed rRT-PCR- positive specimens were aliquoted and refrozen until virus isolation was initiated.

Cell culture, limiting dilution, and isolation

Vero CCL-81 cells were used for isolation and initial passage. Vero E6, Vero CCL-81, HUH 7.0, 293T, A549, and EFKB3 cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with heat inactivated fetal bovine serum (5 or 10%) and anti/anti antibiotic (GIBCO). Both NP an OP swabs were used for virus isolation. For the isolation, limiting dilution, and passage 1 of the virus, 50 µl serum free DMEM was pipetted into columns 2-12 of a 96-well tissue culture plate. One-hundred µl clinical specimens were pipetted into column 1, and then serially diluted 2-fold across the plate. Vero cells were trypsinized and resuspended in DMEM + 10% FBS + 2X Penicillin-Streptomycin + 2X antibiotic – antimycotic + 2 X amphotericin B at 2.5 x 10^5 cells / ml. One hundred µl of cell suspension were added directly to the clinical specimen dilutions and mixed gently by pipetting. The inoculated cultures were grown in a humidified 37°C incubator with 5% CO2 and observed for cytopathic effect (CPE) daily. Standard plaque assays were used for SARS-CoV-2 based on both SARS-CoV and MERS-CoV protocols (16, 17).

When CPE were observed, the cell monolayers were scraped with the back of a pipette tip. Fifty µl of the viral lysate were used for total nucleic acid extraction for confirmatory testing and sequencing. Fifty µl of virus lysate was used to inoculate a well of a 90% confluent 24-well plate.

Inclusivity / Exclusivity testing

From the wells in which CPE were observed, confirmatory testing was performed using CDC's SARS-CoV-2 molecular diagnostic assay (1) The CDC molecular diagnostic assay targets three portions of the N gene, and all three must be positive to be considered as positive result (https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-detection-instructions.html) and (https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html). To confirm that no other respiratory viruses were present, Fast Track respiratory pathogen 33 testing was performed (FTD diagnostics, Silema, Malta).

Whole genome sequencing. Thirty-seven pairs of nested PCR assays spanning the genome were designed based on the reference sequence, Genbank Accession No. NC045512. Nucleic acid was extracted from isolates and amplified by the 37 individual nested PCR assays. Positive PCR amplicons were used individually for subsequent Sanger sequencing and also pooled for library preparation using a ligation sequencing kit (Oxford Nanopore Technologies, Oxford, UK), subsequently for Oxford Nanopore MinION sequencing. Consensus Nanopore sequences were generated using minimap 2.17 and samtools 1.9 (18, 19). Consensus sequences by Sanger sequences were generated from both directions using Sequencher 5.4.6 (Gene Codes Corporation, Ann Arbor, MI), and were further confirmed by consensus sequences generated from nanopore sequencing.

To sequence passage four stock, libraries for sequencing were prepared with the NEB Next Ultra II RNA Prep Kit (New England BioLabs, Inc., Ipswich, MA) following the manufacturer's protocol. Briefly, ~70-100 ng of RNA was fragmented for 15 minutes, followed by cDNA synthesis, end repair and adapter ligation. After 6 rounds of PCR the libraries were analyzed on an Agilent Bioanalyzer and quantified by qPCR. Samples were pooled and sequenced with a paired-end 75 base protocol on an Illumina (Illumina, Inc, San Diego, CA) MiniSeq instrument using the High-Output kit. Reads were processed with Trimmomatic v0.36 (20) to remove low quality base calls and any adapter sequences. The de novo assembly program ABySS (21) was used to assemble the reads into contigs, using several different sets of reads, and kmer values from 20 to 40. Contigs greater than 400 bases long were compared against the NCBI nucleotide collection using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A nearly full length viral contig was obtained in each sample with 100% identity to the 2019-nCoV/USA-WA1/2020 strain (MN985325.1). All the remaining contigs mapped to either host cell ribosomal RNA or mitochondria. The trimmed reads were mapped to the MN985325.1 reference sequence with BWA v0.7.17 (19) and visualized with the Integrated Genomics Viewer (22) to confirm the identity to the USA-WA1/2020 strain.

Electron microscopy

Infected Vero cells were scraped from the flask, pelleted by low speed centrifugation, rinsed with 0.1M phosphate buffer, pelleted again and fixed for 2 hours in 2.5% buffered glutaraldehyde. Specimens were post fixed with 1% osmium tetroxide, *en bloc* staining with 4% uranyl acetate, dehydrated and embedded in epoxy resin. Ultrathin sections were cut, stained with 4% uranyl

acetate and lead citrate, and examined with a Thermo Fisher/FEI Tecnai Spirit electron microscope.

Protein Analysis and Western Blot. Cell lysates were harvested with Laemmli SDS-PAGE sample buffer (Bio RAD, Hercules CA) containing a final concentration of 2% SDS and 5% β-mercaptoethanol. Cell lysates were the boiled and removed from the BSL3. The lysates were then loaded onto a poly-acrylamide gel, and SDS-PAGE followed by transfer to polyvinylidene difluoride PVDF membrane. The membrane was then blocked in 5% nonfat dry milk dissolved in Tris-buffered saline with 0.1% Tween-20 (TBS-T) for 1 hour, followed by a short TBS-T wash. Overnight incubation with primary antibody, either SARS-CoV spike antibody (Sino Biological #40150-T52), β-Actin antibody (Cell Signaling Technology #4970), or a custom SARS-CoV nucleocapsid antibody, was then performed. After primary antibody incubation, the membrane was washed 3x with TBS-T, and then horseradish peroxidase-conjugated secondary antibody was applied for 1 hour. Subsequently, the membrane was washed 3x with TBS-T, and incubated with Clarity Western ECL Substrate (Bio-Rad #1705060S), and imaged with a multi-purpose imaging system.

Generation of SARS Nucleocapsid antibodies. The plasmid, pBM302 (23), was used to express SARS-CoV N protein, with a C-terminal His6 tag, to high levels within the inclusion bodies of E.coli and the recombinant protein was purified from the inclusion bodies by nickel-affinity column chromatography under denaturing conditions. The recombinant SARS-CoV N protein was refolded by stepwise dialysis against Tris/phosphate buffer with decreasing concentrations of urea to renature the protein. The renatured, full-length, SARS-CoV N protein was used to immunize rabbits to generate an affinity-purified rabbit anti-SARS-CoV N polyclonal antibody.

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DISCLAIMERS

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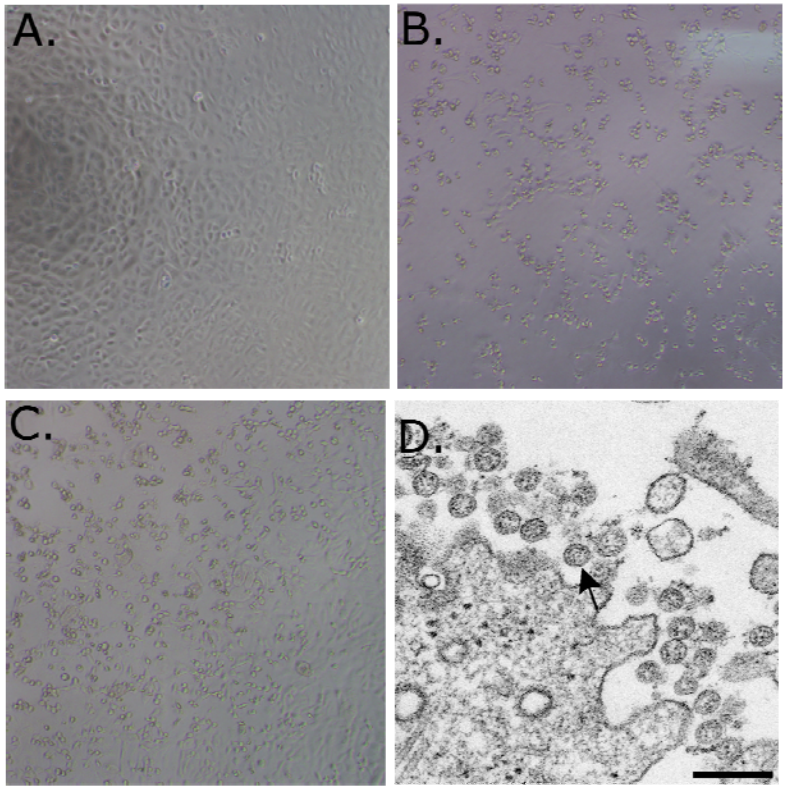

Figure 1.(A-C) 10X phase contrast images of vero monolayers at 3 days post-inoculation. Panels shown are (A) mock, (B) NP, and (C) OP. (D) Electron microscopic image of the viral isolate showing extracellular spherical particles with cross sections through the nucleocapsids, seen as black dots.

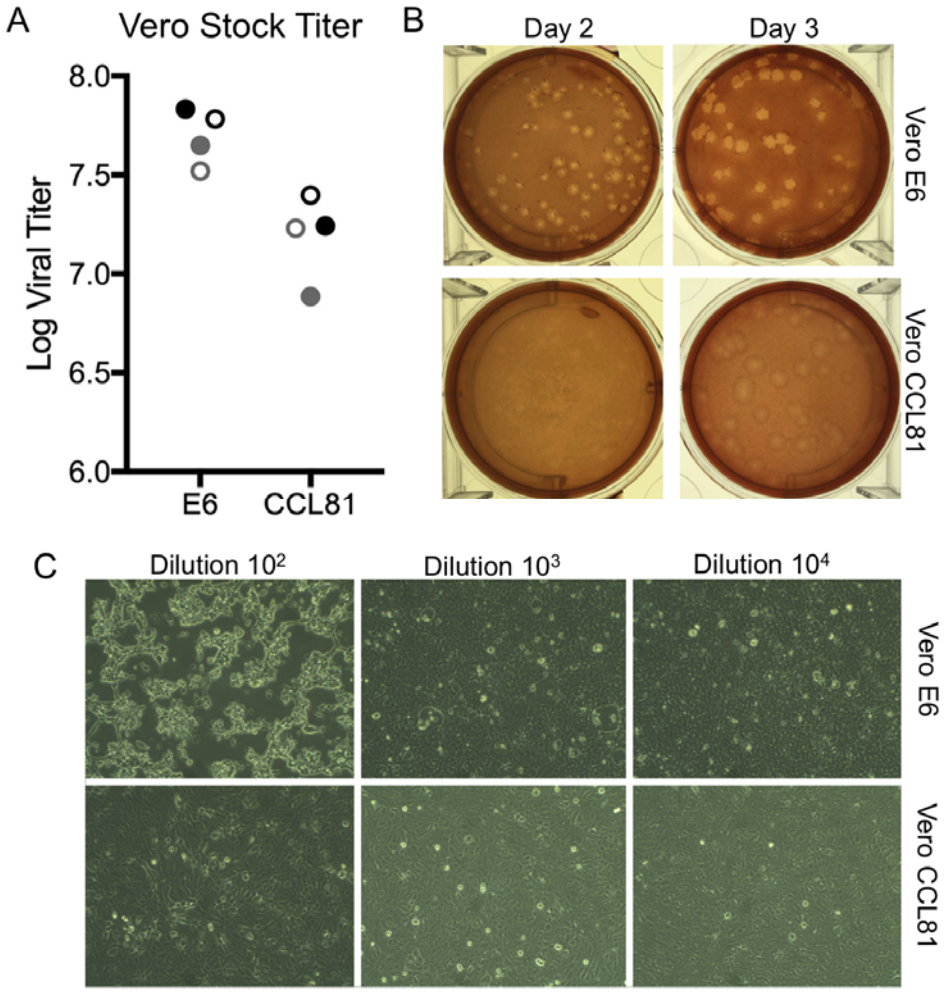

Figure 2. Viral propagation and quantitation. A) Two SARS-CoV-2 passage 4 stocks (black and gray) were quantified utilizing plaque assay at day two (closed circles) and day three (open circles) post infection of Vero E6 and Vero CCL81. B) Plaque morphology on Vero E6 and Vero CCL81 at day 2 and day 3 post inoculation. C) Cell monolayers two days post infection in Vero E6 (top) and Vero CCL81 (bottom row) at multiple dilutions.

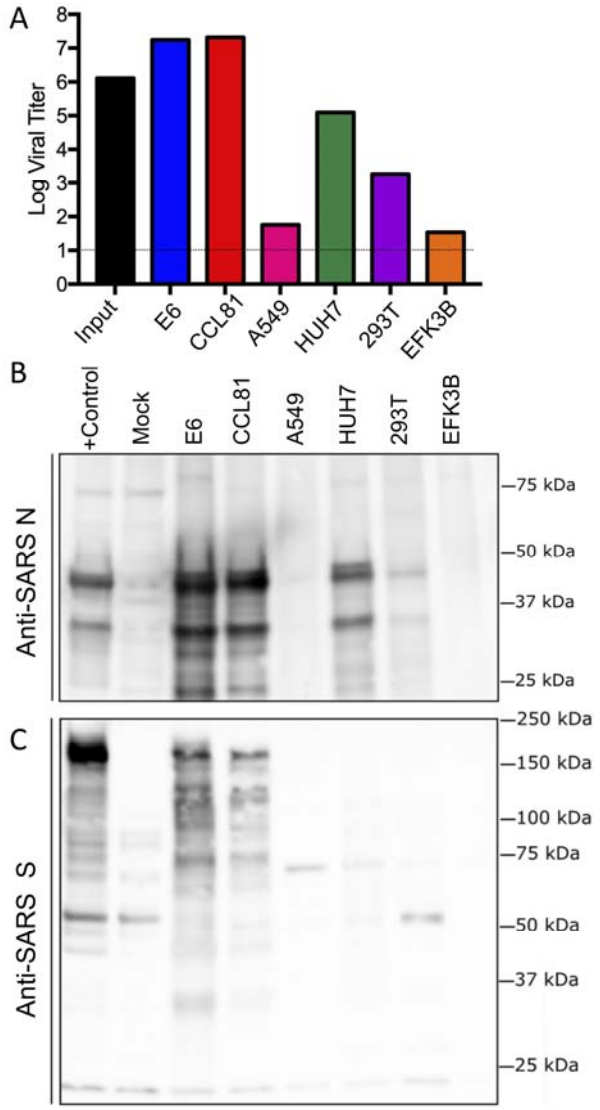

Figure 3. Cell lines susceptible to SARS-CoV-2. (A-C) Cell lines were infected with a high MOI of SARS-CoV-2 (MOI >5), washed after adsorption, and subsequently harvested 24 hours post infection for viral titer and protein lysates. A) Viral titer for SARS-CoV-2 was quantitated by plaque assay on Vero E6 cells two days post inoculation. B & C) Protein lysates were probed by western blot with B) anti-SARS nucleocapsid (N) antibody or C) anti- SARS-CoV spike (S) antibody.