Genomic and phylogenetic characterisation of an imported case of SARS-CoV-2 in Amazonas State, Brazil

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A new coronavirus [severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)] is currently causing a life-threatening pandemic. In this study, we report the complete genome sequencing and genetic characterisation of a SARS-CoV-2 detected in Manaus, Amazonas, Brazil, and the protocol we designed to generate high-quality SARS-CoV-2 full genome data. The isolate was obtained from an asymptomatic carrier returning from Madrid, Spain. Nucleotide sequence analysis showed a total of nine mutations in comparison with the original human case in Wuhan, China, and support this case as belonging to the recently proposed lineage A.2. Phylogeographic analysis further confirmed the likely European origin of this case. To our knowledge, this is the first SARS-CoV-2 genome obtained from the North Brazilian Region. We believe that the information generated in this study may contribute to the ongoing efforts toward the SARS-CoV-2 emergence.

Key words: coronavirus - SARS-CoV-2 - COVID-19 - Brazil - Amazon Region - genome

The coronavirus disease 2019 (COVID-19) is caused by infection with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that was identified for the first time in patients with pneumonia in Wuhan, China.[1,2] Since its discovery at the end of 2019, SARS-CoV-2 transmission has been documented as being person-to-person, causing from an asymptomatic status, that may also generate transmission clusters, to a symptomatic disease with the typical symptoms manifested as fever, dry cough, myalgia, fatigue, dyspnea, diarrhea, and nausea.[3,4] In the majority of the patients, the clinical outcome is a mild disease, although Wu and McGoogan described Chinese patients that developed a severe outcome (16%) or a critical condition (4%). Severe or critical outcomes usually occur in patients with comorbidities, and the disease can progress, presenting arrhythmia and shock, that could evolve to death.[5,6]

The SARS-CoV-2 belongs to family *Coronaviridae*, genus *Betacoronavirus*. In 2002 and 2012, two outbreaks occurred caused by new coronaviruses, SARS-CoV and MERS-CoV, with lethality ranging from nine to 33%, respectively.[7,8,9] The coronaviruses are enveloped viruses, with 80 to 120 nm in diameter. Three proteins compose the virion surface: spike (S), membrane (M), and small membrane protein (E), giving the virus a crown-like visual on electron micrograph.[10,11] This viral family has one of the largest RNA genome of all other RNA viruses, with a single non-segmented positive-stranded RNA of approximately 30 kb.[12]

In Brazil, the first recorded case of SARS-CoV-2 infection occurred in the end of February, in the city of São Paulo, followed by cases in the Northeast (Bahia), Central-West (Brasília) and South (Rio Grande do Sul) regions.[13] The northern of Brazil was the last region of the country to detected SARS-CoV-2 in its population. In March 13th, the first case in the State of Amazonas was detected in a woman that traveled to England and return to the Amazonas capital, Manaus.[14] After three days, the second case of SARS-CoV-2 was detected in Manaus and characterised in the present study.

One 56-years-old man returning from Madrid, Spain, arrived asymptomatic in Manaus, Amazonas State, Brazil on 15-Mar-2020. At that time, a massive outbreak of COVID-19 was already established in several European countries, suggesting that travelers should be kept in

doi: 10.1590/0074-02760200310
Financial support: CNPq, CAPES, MS-DECIT, FIOCRUZ, FAPEAM - REGESAM.
FGN is funded by FAPEAM (PCTI-EmergeSaúde/AM call Nº005/2020 and Rede Genômica de Vigilância em Saúde - REGESAM), CNPq (Grants 440856/2016-7 and 403276/2020-9), CAPES (Grants 88881.130825/2016-01 and 88887.130823/2016-00), Inova Fiocruz/FIOCRUZ (Grant VPPCB-007-FIO-18-2-30 - Geração de conhecimento).
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Received 14 June 2020
Accepted 25 August 2020

quarantine at arrival. Even without the symptoms of a respiratory infection, nasal and oropharyngeal swabs were collected for SARS-CoV-2 testing as a routine established for respiratory virus surveillance at Instituto Leônidas e Maria Deane (ILMD) - Fiocruz, Amazonas State, Brazil, since 2019. The swabs were combined and submitted to total nucleic acid extraction with a commercial kit (Biogene, Recife, PE, Brazil) and immediately evaluated using the reverse transcription real-time polymerase chain reaction (RT-qPCR) protocol developed by the US Centers for Disease Control and Prevention (CDC/USA) (On 15-Mar-2020, CDC updated the RT-qPCR protocol removing the N3 target). This RT-qPCR assay employs different primers and probes sets aiming three regions of the SARS-CoV-2 nucleocapsid (N) gene (https://www.fda.gov/media/134922/download), and the human RNase P as an internal control.

The analysed sample tested positive for SARS-CoV-2 with Cts values of 14.43 (N1), 15.39 (N2) and15.33 (N3). Thus, we immediately generated cDNA with random primers and Superscript IV reverse transcriptase (ThermoFisher Scientific, Waltham, MA, United States). We had previously designed a PCR scheme to amplify the entire genome of the SARS-CoV-2 based on an alignment of all complete genome sequences available on GenBank at 03/03/2020. Conserved regions were chosen for primer design with Primer3 v2.3.7. embedded in Geneious Software 10.2.6,[15] spanning around 2.2Kb and with an overlap region between 131 and 225 bp. This PCR scheme resulted in 15 amplicons with further details presented in the **Supplementary data (Table I)**.

The SARS-CoV-2 whole genome was amplified with Platinum SuperFi II Green PCR master mix (ThermoFisher Scientific) using the 15 primers sets in individual reactions. Each amplicon was then visualised as a unique and intense DNA fragment on agarose gel electrophoresis stained with GelRed (Biotium, Hayward, CA, United States). The PCR amplicons were precipitated with molecular biology grade Polietilenoglicol (PEG) 8,000 (Promega, Madison, WI, United States) and then resuspended in nuclease-free water. After one-hour incubation at 37ºC, all amplicons were quantified in ng/µL using Qubit 2.0 and the dsDNA HS assay kit (Thermo Fisher Scientific). Finally, the number of DNA copies in each purified amplicon was estimated with ENDMEMO (http://www.endmemo.com/bio/dnacopynum.php), normalised, and pooled. A single library was constructed using the Nextera DNA Flex Library Prep and clustered with MiSeq Reagent Micro Kit v2 (300-cycles), following the manufacturer's protocols. Nucleotide sequencing was performed in the MiSeq platform (Illumina, San Diego, CA, United States), installed at ILMD, in a paired-end run (2x150 cycles).

A total of 10,946,898 reads were trimmed for quality and adapters using BBDUK v37.25, embedded in Geneious software. Thus, 8,362,418 reads were mapped to the SARS-CoV-2 NCBI Reference Sequence NC_045512.2 using Geneious map-to-reference tool. The BR_AM_ILMD_20140001 final consensus genome sequence contains 29,789 nucleotides, with no gaps, a Q40 score of 100%, with no undetermined "N" bases and high average coverage (> 34,000X). To avoid any primers bias, we removed both primer binding sites at the 5′ and 3′ ends. Thus, our final sequence represents the positions between nucleotides 47 and 29,835 or 99.6% of the NCBI RefSeq previously mentioned.

We aligned the BR_AM_ILMD_20140001 genomic sequence with the SARS-CoV-2 NCBI Reference Sequence NC_045512 using MAFFT v7.388[16] to investigate any mutations throughout genome. A total of nine mutations were observed at nucleotide positions 8,782 (C to T); 9,477 (T to A); 12,781 (C to T); 14,805 (C to T); 25,979 (G to T); 26,642 (C to T); 28,144 (T to C); 28,657 (C to T) and 28,863 (C to T), with four of these leading to residues substitution in the deduced protein sequences (Table).

To put the BR_AM_ILMD_20140001 genome in a global context, we aligned the new genome to a pool of all SARS-CoV-2 genomes with at least 25,000 nucleotides available at GISAID database[17] on March 31st, 2020 using MAFFT. We adopted a subsampling strategy to reduce

TABLE

Differences observed between sample BR_AM_ILMD_20140001 and the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) prototype

Genome position	8782	9477	12781	14805	25979	26642	28144	28657	28863
NC_045512	C	T	C	C	G	C	T	C	C
BR_AM_ILMD	T	A	T	T	T	T	C	T	T
Codon position	AG**C**	T**T**T	TA**C**	TA**C**	GGA	GCC	T**T**A	GA**C**	T**C**A
	AG**T**	TA**T**	TA**T**	TA**T**	GTA	GCT	T**C**A	GA**T**	T**T**A
Mutation type	s	ns	s	s	ns	s	ns	s	ns
Protein	nsp4	nsp4	nsp9	RdRp	ORF3a ptn	M glycoptn	ORF8 ptn	npptn	npptn
Residue	Ser2839	Phe3071Tyr	Tyr4172	Tyr4847	Gly196Val	Ala40	Leu84Ser	Asp128	Ser197Leu

Nucleotides substituted in each codon are represented in bold. s: silent mutation; ns: non-silent mutation; nsp: non-structural protein; RdRp: RNA-dependent RNA-polymerase; ORF3a ptn: ORF3a protein; M glycoptn: M glycoprotein; ORF8 ptn: ORF8 protein; npptn: nucleocapsid phosphoprotein.

computation time, selecting subsets of 15-20 sequences retaining the most viral diversity from each country using the CD-HIT program.[18] This subsampling approach resulted in a final sequence dataset with 490 sequences from 53 countries [**Supplementary data (Table II)**].

Complete coding sequences (CDS) were subjected to maximum likelihood (ML) phylogenetic reconstruction with PhyML v3.0,[19] under the HKY+Γ4 nucleotide substitution model. According to the ML phylogenetic tree, the BR_AM_ILMD_20140001 sequence belonged to the lineage A[20] (Fig. 1A) clustering within a monophyletic cluster (aLRT = 1.00) comprising sequences from Spain, Chile, France, Greece, Georgia, Netherlands, Senegal (Fig. 1B). The pangolin web application (pangolin.cog-uk.io) further assigned the sequences from this cluster to the A.2 lineage (Fig. 1B).

After temporal validation with Tempest[21] (Fig. 2A), we conducted a Bayesian discrete spatiotemporal analysis with all lineage A sequences (Fig. 2) using the BEAST v1.10 package,[22] applying the strict molecular clock and the parametric exponential coalescent models. The strict molecular clock model was selected over the relaxed uncorrelated one,[23] after marginal likelihood estimation using path sampling and stepping-stone sampling methods[24] [**Supplementary data (Table III)**].The analysis under the uncorrelated relaxed molecular clock model, however, resulted in estimates similar to those of the strict clock model (data not shown). We estimated that the most recent common ancestor (MRCA) of lineage A.2 originated in Spain (posterior state probability, PSP = 0.99) in the beginning of February 2020 (95% highest posterior density, HPD = 2nd - 22nd Feb 2020). The phylogeographic analysis also pointed out that the BR_AM_ILMD_20140001 was most probably introduced from Spain (PSP = 0.43) (Fig. 2B).

Like other viral infections, the infection by SARS-CoV-2 can be asymptomatic and this fact has been reported in different studies.[25,26,27] It is noteworthy that according to the definition of suspected case adopted in Brazil at the time that we investigated this asymptomatic carrier, he would not be included in SARS-CoV-2 testing, despite returning from an area with active transmission like Madrid, Spain.[28] Until 24-Mar-2020, when we deposited the BR_AM_ILMD_20140001 genome information at https://www.gisaid.org, there were only 17 Brazilian SARS-CoV-2 complete genome sequences, and the sequence reported in the present work is the first complete genome from the northern Brazilian region.

Several laboratories over the world are now sequencing thousands of SARS-CoV-2 genomes, which is undoubtedly the most notable effort of viral sequencing in human history. Like any other virus, the new coronavi-

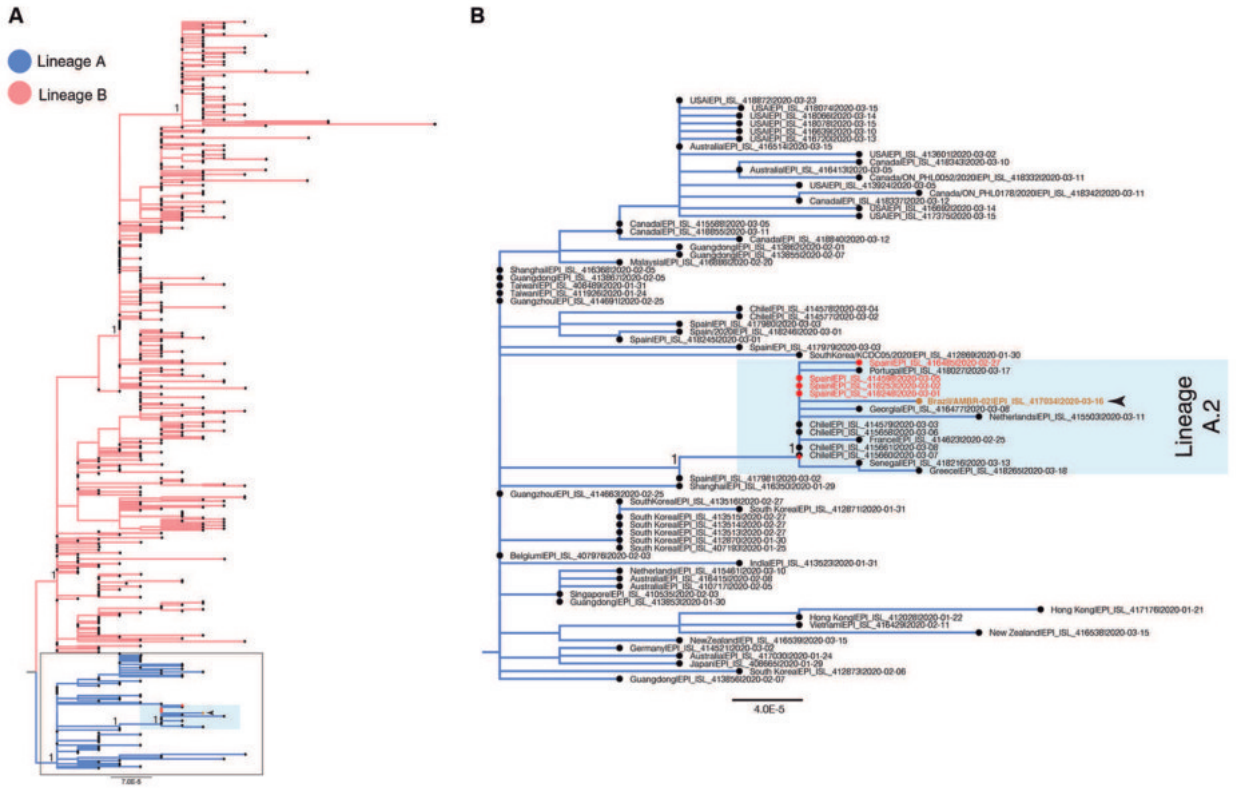

Fig. 1: maximum-likelihood (ML) phylogeny of subsampled severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) genomes. (A) ML tree rooted on the branch separating lineages A and B sequences colored following the legend. (B) A close view of the lineage A highlighting the A.2 lineage (light blue box) that comprises the BR_AM_ILMD_20140001 strain (indicated with an arrow). The nodes representing the most recent common ancestor (MRCA) of the lineage A.2 is indicated with a red diamond. In both trees, tips representing the Spanish strains inside lineage A.2 are coloured red and the scale bar represents nucleotide substitutions per site.

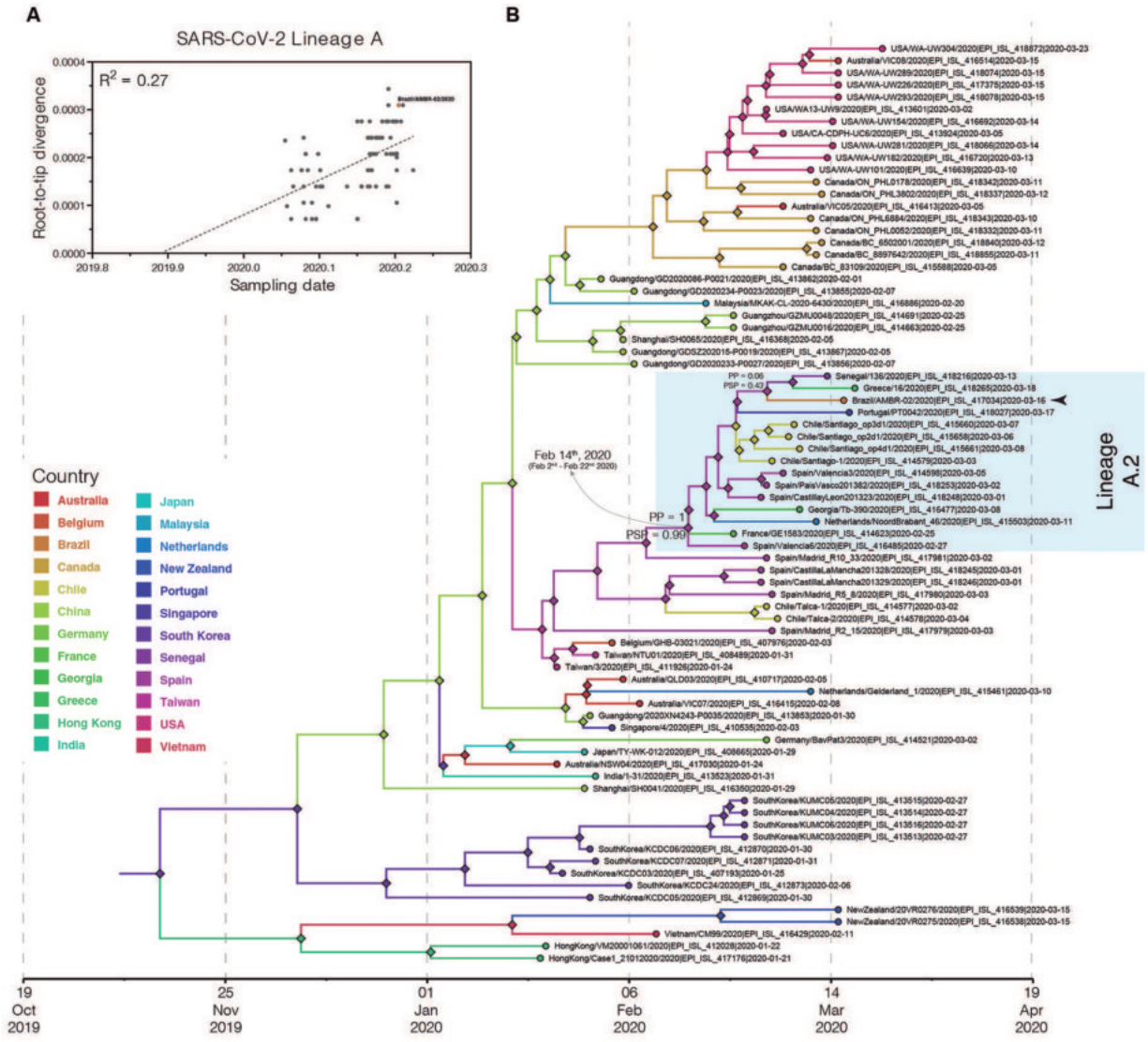

Fig. 2: phylogeography of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) lineage A. (A) Temporal signal analysis correlating the sampling date of each sequence and its genetic distance from the root of a maximum likelihood phylogeny. (B) Time-scaled Bayesian phylogeographic maximum clade credibility (MCC) tree of the SARS-CoV-2 complete coding sequences (CDS) classified as lineage A. The branch's colors represent the most probable location of their descendent nodes (diamonds) as indicated at the legend. Branch support are indicated only at key nodes [posterior (PP) and posterior state probability (PSP)]. The lineage A.2 is highlighted with a light blue box. All horizontal branch lengths are drawn to a scale of years.

rus is continuously evolving as more hosts, humans or animals, are getting infected. Thus, it is of paramount importance to generate and share high-quality full viral genomes from different regions over the world to better understand the SARS-CoV-2 evolution. The information related to the viral evolution is not only necessary for molecular epidemiology studies, but also to monitor if the newly identified mutations are linked to different clinical presentations or may drive into false-negative results when performing nucleic acid amplification assays, like real-time PCR.

Therefore, in this work, we aimed to describe and characterise the complete genome of the SARS-CoV-2 obtained from an asymptomatic carrier returning from Madrid, Spain. To achieve this goal, we decided to use a nucleotide sequencing strategy where firstly all the 15 amplicons, encompassing the entire SARS-CoV-2 genome, were confirmed by agarose gel electrophoresis. Subsequently, each amplicon was quantified and normalised in order to prevent that one region could be overrepresented during sequencing. In order to make our approach more straightforward, we decided to evaluate if longer amplicons could be generated in a very similar way. We were successful in generating amplicons around 6 Kb, with a minimum overlap of 131 bp, reducing the number of PCR reactions to 5 instead of 15 (data not showed). The conditions to amplify the 6 Kb amplicons followed the manufacturer's recommendations, with the primers pairs details presented in the **Supplementary data (Table I)** of this manuscript. Of

note, we observed that only samples with high viral load (e.g. Ct lower than 21) were fully amplified using the 6Kb protocol. We believe that this approach may be exciting not only to reduce the current protocol costs, but also for those interested in using long reads sequencing technologies like PacBio SMS and nanopore.

Recently, Rambaut and colleagues proposed a rational and dynamic virus classification for SARS-CoV-2 genomes based on a phylogenetic framework.[20] Using this approach authors identified at the root of the phylogeny of SARS-CoV-2 two lineages that were simply denoted as lineages A and B. In our analysis, while all Brazilian SARS-CoV-2 sequences belonged to the lineage B, mostly from the B.1 lineage, the BR_AM_ILMD_20140001 genome clustered within the lineage A.2, indicating that BR_AM_ILMD_20140001 strain belongs to a distinct transmission cluster than the other full Brazilian genomes reported until March 31st, 2020. Originally, the lineage B.1 predominated in Europe and North America,[17,20] and currently, constitutes the most prevalent SARS-CoV-2 variant circulating in Brazil.[29] The lineage A.2 constitutes a predominantly Spanish lineage, found in at least 25 countries[20] and our phylogeographic analysis corroborates its origins and reinforces the importation scenario from Spain to Brazil.

In this study, we report and characterise the first SARS-CoV-2 genome obtained from an infected subject in the Brazilian North Region. Since this case was an asymptomatic carrier, it is not easy to suggest when infection has occurred. However, our phylogeographic analysis strongly indicates this individual was infected in Spain. Finally, we would like to emphasise that more fully genomes studies of the SARS-CoV-2 are necessary to better understand the evolution of this emerging life-threatening virus and the information of the nucleotide sequence described here may contribute to future molecular epidemiological studies in Brazil. In this sense, the protocol that we described in the present study may be useful to aid other researchers to generate other high-quality SARS-CoV-2 genomes.

Nucleotide sequence accession number - The complete genome sequence of the BR_AM_ILMD_20140001 isolate is available in GISAID since March 24, 2020, under the ID number EPI_ISL_417034.

ACKNOWLEDGEMENTS

To the following authors from the originating laboratories responsible for obtaining the specimens and the submitting laboratories where genetic sequence data were generated and shared via the GISAID Initiative, on which this research is based. All GISAID accession numbers and data contributors are described in the Supplementary data (Table IV). The authors thank the Program for Technological Development in Tools for Health - FIOCRUZ - for use of nucleotide sequencing facilities at ILMD - Fiocruz Amazônia.

AUTHORS' CONTRIBUTION

VAN, SLBL, CFC and FGN conceived the study; FGN designed the study protocol; VAN, ALGC, FON, AKAC, DCGD, LMFG, MSJ and FGN performed the molecular tests; VAN, ED and FGN performed the analysis and interpretation of these data; FON and MSJ collected biological sample; VAN, ALGC, AKAC, FON, DCGD, LMFG, ED and FGN wrote the manuscript; SLBL, CFC, ED and FGN critically revised the manuscript for intellectual content; FGN financed the study. All authors read and approved the final manuscript. . The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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RESEARCH

Severe Acute Respiratory Syndrome Coronavirus 2 from Patient with Coronavirus Disease, United States

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The etiologic agent of an outbreak of pneumonia in Wuhan, China, was identified as severe acute respiratory syndrome coronavirus 2 in January 2020. A patient in the United States was given a diagnosis of infection with this virus 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 from this patient and characterized the viral sequence, replication properties, and cell culture tropism. We found that the virus replicates to high titer in Vero-CCL81 cells and Vero E6 cells in the absence of trypsin. We also deposited the virus into 2 virus repositories, making it broadly available to the public health and research communities. We hope that open access to this reagent will expedite development of medical countermeasures.

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DOI: https://doi.org/10.3201/eid2606.200516

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 β 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 of coronaviruses mediates virus binding and cell entry. Initial characterization of SARS-CoV-2 spike indicates that it binds the same receptor as SARS-CoV angiotensin-converting enzyme, which is expressed in both upper and lower human respiratory tracts (*8*).

The unprecedented rapidity of spread of this outbreak represents a critical need for reference reagents. The public health community requires viral lysates to serve as diagnostic references, and the research community needs virus isolates to test antiviral compounds, develop new vaccines, and perform basic research. In this article, we describe isolation of SARS-CoV-2 from a patient who had coronavirus disease (COVID-19) in the United States and described its genomic sequence and replication characteristics. We have made the virus isolate available to the public health community by depositing it into 2 virus reagent repositories.

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Methods

Specimen Collection

Virus isolation from patient samples was deemed not to be human subjects research by the National Center for Immunizations and Respiratory Diseases, Centers for Disease Control and Prevention (CDC) (research determination no. 0900f3eb81ab4b6e). Clinical specimens from a case-patient who had acquired COVID-19 during travel to China and who was identified in Washington, USA, were collected as described (*1*). Nasopharyngeal (NP) and oropharyngeal (OP) swab specimens were collected on day 3 postsymptom onset, placed in 2–3 mL of viral transport medium, used for molecular diagnosis, and frozen. Confirmed PCR-positive specimens were aliquoted and refrozen until virus isolation was initiated.

Cell Culture, Limiting Dilution, and Virus Isolation

We used Vero CCL-81 cells for isolation and initial passage. We cultured Vero E6, Vero CCL-81, HUH 7.0, 293T, A549, and EFKB3 cells in Dulbecco minimal essential medium (DMEM) supplemented with heat-inactivated fetal bovine serum (5% or 10%) and antibiotics/antimycotics (GIBCO, https://www.thermofisher.com). We used both NP and OP swab specimens for virus isolation. For isolation, limiting dilution, and passage 1 of the virus, we pipetted 50 μL of serum-free DMEM into columns 2–12 of a 96-well tissue culture plate, then pipetted 100 μL of clinical specimens into column 1 and serially diluted 2-fold across the plate. We then trypsinized and resuspended Vero cells in DMEM containing 10% fetal bovine serum, 2× penicillin/streptomycin, 2× antibiotics/antimycotics, and 2× amphotericin B at a concentration of 2.5×10^5 cells/mL. We added 100 μL of cell suspension directly to the clinical specimen dilutions and mixed gently by pipetting. We then grew the inoculated cultures in a humidified 37°C incubator in an atmosphere of 5% CO_2 and observed for cytopathic effects (CPEs) daily. We used standard plaque assays for SARS-CoV-2, which were based on SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV) protocols (*9*,*10*).

When CPEs were observed, we scraped cell monolayers with the back of a pipette tip. We used 50 μL of viral lysate for total nucleic acid extraction for confirmatory testing and sequencing. We also used 50 μL of virus lysate to inoculate a well of a 90% confluent 24-well plate.

Inclusivity/Exclusivity Testing

From the wells in which CPEs were observed, we performed confirmatory testing by using real-time reverse transcription PCR (CDC) and full-genome sequencing (*1*). The CDC molecular diagnostic assay targets 3 portions of the nucleocapsid gene, and results for all 3 portions must be positive for a sample to be considered positive (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, we performed Fast Track Respiratory Pathogens 33 Testing (FTD Diagnostics, http://www.fast-trackdiagnostics.com).

Whole-Genome Sequencing

We designed 37 pairs of nested PCRs spanning the genome on the basis of the coronavirus reference sequence (GenBank accession no. NC045512). We extracted nucleic acid from isolates and amplified by using the 37 individual nested PCRs. We used positive PCR amplicons individually for subsequent Sanger sequencing and also pooled them for library preparation by using a ligation sequencing kit (Oxford Nanopore Technologies, https://nanoporetech.com), subsequently for Oxford Nanopore MinION sequencing. We generated consensus nanopore sequences by using Minimap version 2.17 (https://github.com) and Samtools version 1.9 (http://www.htslib.org). We generated consensus sequences by Sanger sequencing from both directions by using Sequencher version 5.4.6 (https://www.genecodes.com), and further confirmed them by using consensus sequences generated from nanopore sequencing.

To sequence passage 4 stock, we prepared libraries for sequencing by using the Next Ultra II RNA Prep Kit (New England Biolabs, https://www.neb.com) according to the manufacturer's protocol. In brief, we fragmented ≈70–100 ng of RNA for 15 min, followed by cDNA synthesis, end repair, and adaptor ligation. After 6 rounds of PCR, we analyzed libraries by using an Agilent Bioanalyzer (https://www.agilent.com) and quantified them by using a quantitative PCR. We pooled samples and sequenced samples by using a paired-end 75-base protocol on an Illumina (Illumina, Inc., https://www.illumina.com) MiniSeq instrument and using the High-Output Kit and then processed reads by using Trimmomatic version 0.36 (*11*) to remove low-quality base calls and any adaptor sequences. We used the de novo assembly program ABySS (*12*) to assemble the reads into contigs by using several different sets of reads and kmer values ranging from 20 to 40. We compared contigs >400 bases against the National Center for Biotechnology Information (Bethesda, MD, USA) nucleotide collection using BLAST

(https://blast.ncbi.nlm.nih.gov). A nearly full-length viral contig obtained in each sample had 100% identity to the 2019-nCoV/USA-WA1/2020 strain (GenBank accession no. MN985325.1). All the remaining contigs mapped to either host cell rRNA or mitochondria. We mapped the trimmed reads to the reference sequence by using BWA version 0.7.17 (*13*) and visualized these reads by using the Integrated Genomics Viewer (*14*) to confirm the identity with the USA-WA1/2020 strain.

Electron Microscopy

We scraped infected Vero cells from the flask, pelleted by low-speed centrifugation, rinsed with 0.1 mol/L phosphate buffer, pelleted again, and fixed for 2 h in 2.5% buffered glutaraldehyde. We then postfixed specimens with 1% osmium tetroxide, en bloc stained with 4% uranyl acetate, dehydrated, and embedded in epoxy resin. We cut ultrathin sections, stained them with 4% uranyl acetate and lead citrate, and examined them by using a Thermo Fisher/FEI Tecnai Spirit electron microscope (https://www.fei.com).

Protein Analysis and Western Blotting

We harvested cell lysates by using Laemmli sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer (Bio-Rad, https://www.bio-rad.com) containing 2% SDS and 5% β-mercaptoethanol. We removed the cell lysates from a Biosafety Level 3 Laboratory, boiled them, and loaded them onto a polyacrylamide gel. We subjected the lysates to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by transfer to a polyvinylidene difluoride polyvinylidene fluoride membrane. We then blocked the membrane in 5% nonfat dry milk dissolved in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 h, followed by a short wash with TBS-T. We incubated the membrane overnight with primary antibody, either rabbit polyclonal serum against the SARS-CoV spike protein (#40150-T52; Sino Biological, https://www.sinobiological.com), β-actin antibody (#4970; Cell Signaling Technology, https://www.cellsignal.com), or a custom rabbit polyclonal serum against SARS-CoV nucleocapsid. We then washed the membrane with 3 times with TBS-T and applied horseradish peroxidase-conjugated secondary antibody for 1 h. Subsequently, we washed the membrane 3 times with TBS-T, incubated with Clarity Western ECL Substrate (#1705060S; Bio-Rad), and imaged with a multipurpose imaging system.

Generation of SARS-CoV Nucleocapsid Antibodies

We used the plasmid pBM302 (*15*) to express SARS-CoV nucleocapsid protein, with a C-terminal His6 tag, to high levels within the inclusion bodies of *Escherichia coli* and the recombinant protein was purified from the inclusion bodies by using nickel-affinity column chromatography under denaturing conditions. We used stepwise dialysis against Tris/phosphate buffer to refold the recombinant SARS-CoV nucleocapsid protein with decreasing concentrations of urea to renature the protein. We then immunized rabbits with the renatured, full-length, SARS-CoV nucleocapsid protein to generate an affinity-purified rabbit anti–SARS-CoV nucleocapsid protein polyclonal antibody.

Results

A patient was identified with confirmed COVID-19 in Washington State on January 22, 2020. CPE was not observed in mock infected cells (Figure 1, panel A). Cycle threshold (C_t) values were 18–20 for NP specimens and 21–22 for OP specimens (*1*). The positive clinical specimens were aliquoted and refrozen inoculated into cell culture on January 22, 2020. We observed CPE 2 days postinoculation and harvested viral lysate on day 3 postinoculation (Figure 1, panels B, C). We used 50 μL of passage 1 viral lysates for nucleic acid extraction to confirm the presence of SARS-CoV-2 by using the CDC molecular diagnostic assay (*1*). The C_t values of 3 nucleic acid extractions were 16.0–17.1 for nucleocapsid portion 1, 15.9–17.1 for nucleocapsid portion 2, and 16.2–17.3 for nucleocapsid portion 3, which confirmed isolation of SARS-CoV-2 (C_t <40 is considered a positive result). We also tested extracts for 33 additional different respiratory pathogens by using the Fast Track 33 Assay. No other pathogens were detected. Identity was additionally supported by thin-section electron microscopy (Figure 1, panel D). We observed a morphology and morphogenesis characteristic of coronaviruses.

We used isolates from the first passage of an OP and an NP specimen for whole-genome sequencing. The genomes from the NP specimen (GenBank accession MT020880) and OP specimen (GenBank accession no. MT020881) showed 100% identity with each other. The isolates also showed 100% identity with the corresponding clinical specimen (GenBank accession no. MN985325).

After the second passage, we did not culture OP and NP specimens separately. We passaged virus isolate 2 more times in Vero CCL-81 cells and titrated by determining the 50% tissue culture infectious dose ($TCID_{50}$). Titers were 8.65×10^6 $TCID_{50}$/mL for the third passage and 7.65×10^6 $TCID_{50}$/mL for the fourth passage.

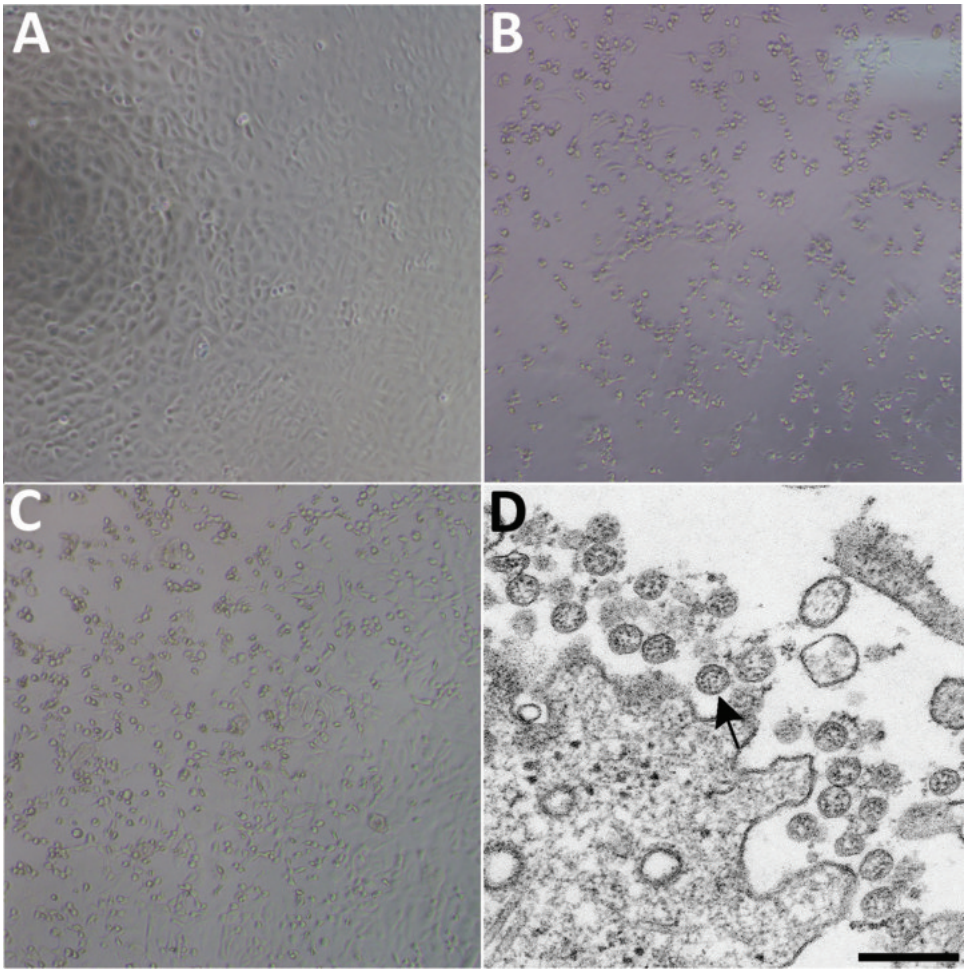

Figure 1. Cytopathic effect caused by severe acute respiratory syndrome coronavirus 2 from patient with coronavirus disease, United States, 2020. A–C) Phase-contrast microscopy of Vero cell monolayers at 3 days postinoculation: A) Mock, B) nasopharyngeal specimen, C) oropharyngeal specimen. Original magnifications ×10). D) Electron microscopy of virus isolate showing extracellular spherical particles with cross-sections through the nucleocapsids (black dots). Arrow indicates a coronavirus virion budding from a cell. Scale bar indicates 200 nm.

We passaged this virus in the absence of trypsin. The spike protein sequence of SARS-CoV-2 has an RRAR insertion at the S1-S2 interface that might be cleaved by furin (*16*). 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 replication (*17*). The RRAR insertion in SARS-CoV-2 might 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. We sequenced viral RNA from SARS-CoV-2 passage 4 stock and confirmed it to have no nucleotide mutations compared with the original reference sequence (GenBank accession no. MN985325). SARS-CoV has been found to grow well on VeroE6 cells and MERS-CoV on Vero CCL81 cells (*18,19*). To establish a plaque assay and determine the preferred Vero cell type for quantification, we titered our passage 4 stock on VeroE6 and VeroCCL81 cells. After infection with a dilution series, SARS-CoV-2 replicated in both Vero cell types; however, the viral titers were slightly higher in VeroE6 cells than in Vero CCL81 cells (Figure 2, panel A). In addition, plaques were more distinct and visible on Vero E6 cells (Figure 2, panel B). As early as 2 days postinoculation, VeroE6 cells produced distinct plaques visible by staining with neutral red. In contrast, Vero CCL81 cells produced less clear plaques and was most easily quantitated by staining with neutral red 3 days postinoculation. On the individual plaque monolayers, SARS-CoV-2 infection of Vero E6 cells produced CPE with areas of cell clearance (Figure 2, panel C). In contrast, Vero CCL81 cells had areas of dead cells that had fused to form plaques, but the cells did not clear. Together, these results suggest that VeroE6 cells might be the best choice for amplification and quantification, but both Vero cell types support amplification and replication of SARS-CoV-2.

Because research has been initiated to study and respond to SARS-CoV-2, information about cell lines and types susceptible to infection 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

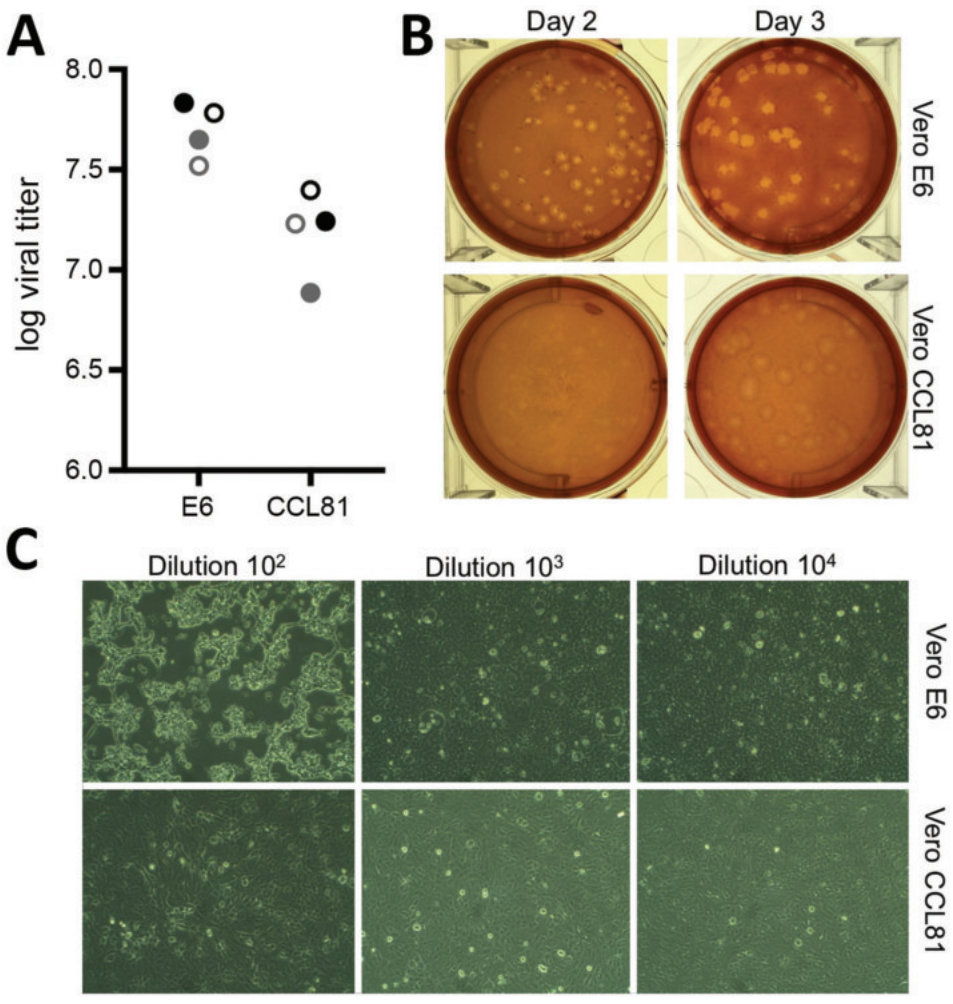

Figure 2. Viral propagation and quantitation of severe acute respiratory syndrome coronavirus 2 from patient with coronavirus disease, United States, 2020. A) Two virus passage 4 stocks (black and gray circles) were quantified by using plaque assay at day 2 (solid circles) and day 3 (open circles) postinfection of Vero E6 and Vero CCL81 cells. B) Plaque morphology for virus on Vero E6 and Vero CCL81 at day 2 and day 3 postinoculation. C) Cell monolayers 2 days postinfection of Vero E6 (top) and Vero CCL81 (bottom) at 3 dilutions. Original magnifications ×40.

cells (A549), human liver cells (HUH7.0), and human embryonic kidney cells (HEK-293T), in addition to Vero E6 and Vero CCL81 cells. We also examined an available big brown bat kidney cell line (EFK3B) for SARS-CoV-2 replication capacity. Each cell line was inoculated at high multiplicity of infection and examined 24 h postinfection (Figure 3, panel A). No CPE was observed in any of the cell lines except in Vero cells, which grew to >10^7 PFU at 24 h postinfection. In contrast, 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 and suggest other common culture systems, including MDCK, HeLa, HEP-2, MRC-5 cells, and embryonated eggs, are unlikely to support SARS-CoV-2 replication (*20*–*22*). In addition, SARS-CoV-2 did not replicate in bat EFK3B cells, which are susceptible to MERS-CoV. Together, the results indicate that SARS-CoV-2 maintains 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 polyclonal serum against the SARS-CoV spike protein and nucleocapsid proteins recognize SARS-CoV-2 (Figure 3, panels B, C). The nucleocapsid protein, which is 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 3, panel A), SARS-CoV-2 showed robust nucleocapsid protein in both Vero cell types, less protein in HUH7.0 and 293T cells, and minimal protein in A549 and EFK3B cells (Figure 3, panel B). The SARS-CoV spike protein antibody also recognized SARS-CoV-2 spike protein, indicating cross-reactivity (Figure 3, panel C). Consistent with SARS CoV, several cleaved and uncleaved forms of the SARS-CoV-2 spike protein were observed. The cleavage pattern of the SARS spike positive control from Calu3 cells, a respiratory

cell line, varies slightly and could indicate differences between proteolytic cleavage of the spike proteins between the 2 viruses because of a predicted insertion of a furin cleavage site in SARS-CoV-2 (*16*). However, differences in cell type and conditions complicate this interpretation and indicate the need for further study in equivalent systems. Overall, the protein expression data from SARS-CoV nucleocapsid and spike protein antibodies recapitulate replication findings and indicate that SARS-CoV reagents can be used to characterize SARS-CoV-2 infection.

Finally, we evaluated the replication kinetics of SARS-CoV-2 in a multistep growth curve. In brief, we infected Vero CCL-81 and HUH7.0 cells with SARS-CoV-2 at a low multiplicity of infection (0.1) and evaluated viral replication every 6 h for 72 h postinoculation, with separate harvests in the cell-associated and supernatant compartments (Figure 4). Similar to SARS-CoV, SARS-CoV-2 replicated rapidly in Vero cells after an initial eclipse phase, achieving 10^5 $TCID_{50}$/mL by 24 h postinfection and peaking at >10^6 $TCID_{50}$/mL. We observed similar titers in cell-associated and supernatant compartments, which indicated efficient egress. Despite peak viral titers by 48 h postinoculation, major CPE was not observed until 60 h postinoculation and peaked at 72 h postinoculation, indicating that infected monolayers should be harvested before peak CPE is observed. Replication in HUH7.0 cells also increased quickly after an initial eclipse phase but plateaued by 24 h postinoculation in the intracellular compartment at 2 × 10^3 $TCID_{50}$/mL and decreased after 66 h postinoculation. Virus was not detected in the supernatant of infected HUH7 cells until 36 h postinoculation and exhibited lower titers at all timepoints (Figure 4). Major CPE was never observed in HUH7.0 cells. These results are consistent with previous reports for SARS-CoV and MERS-CoV, which suggested similar replication dynamics between the zoonotic CoV strains (*23*,*24*).

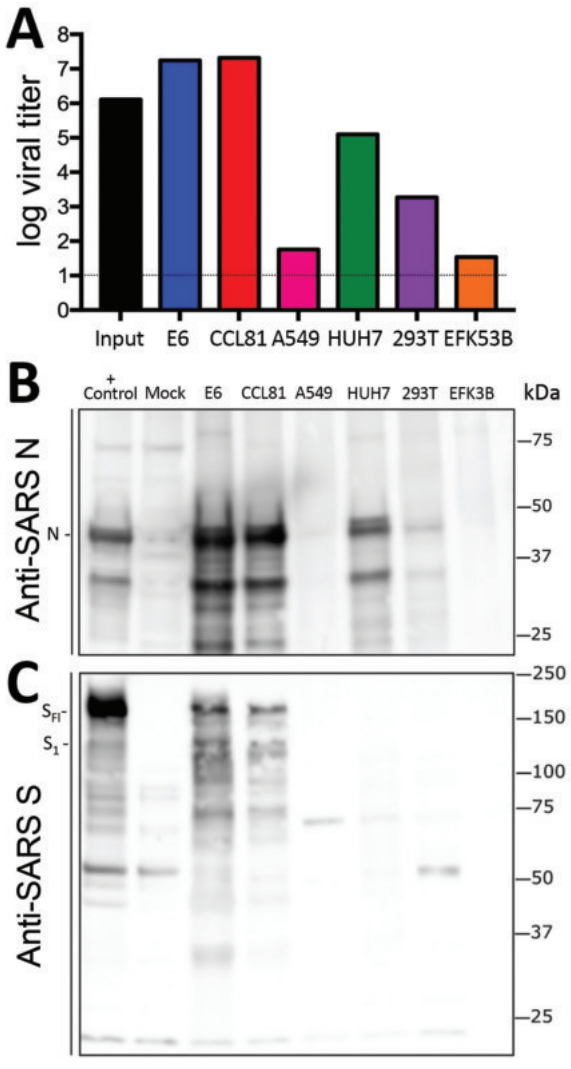

Figure 3. Cell lines from patient with coronavirus disease, United States, 2020, susceptible to SARS coronavirus 2 (SARS-CoV-2). Cell lines were infected with a high multiplicity of infection (>5), washed after adsorption, and subsequently harvested 24 h postinfection for viral titer and protein lysates. A) Viral titer for SARS-CoV-2 quantitated by plaque assay on Vero E6 cells 2 days postinoculation. Infected cell protein lysates were probed by using Western blotting with B) rabbit polyclonal anti-SARS N antibody or C) anti–SARS-CoV S protein antibody. Full-length spike protein (S_{FL}) and spike protein S1 (S_1) are indicated. N, nucleocapsid; S, spike protein; SARS, severe acute respiratory syndrome.

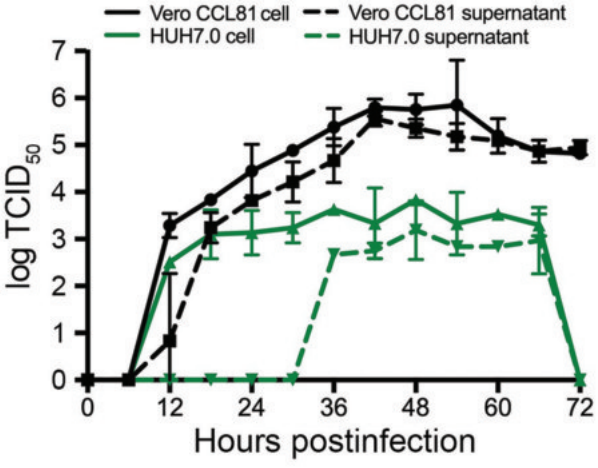

Figure 4. Multistep growth curve for severe acute respiratory syndrome coronavirus 2 from patient with coronavirus disease, United States, 2020. Vero CCL81 (black) and HUH7.0 cells (green) were infected at a multiplicity of infection of 0.1, and cells (solid line) and supernatants (dashed line) were harvested and assayed for viral replication by using $TCID_{50}$. Circles, Vero CCL81 cells; squares, Vero CCL81 supernatants; triangles, HUH7.0 cells; inverted triangles, HUH7.0 supernatants. Error bars indicate SEM. $TCID_{50}$, 50% tissue culture infectious dose.

Discussion

We have deposited information on the SARS-CoV-2 USA-WA1/2020 viral strain described here into the Biodefense and Emerging Infections Research Resources Repository (https://www.beiresources.org) reagent resources (American Type Culture Collection, https://www.atcc.org) and the World Reference Center for Emerging Viruses and Arboviruses, University of Texas Medical Branch (https://www.utmb.edu/wrceva), to serve as the SARS-CoV-2 reference strain for the United States. The SARS-CoV-2 fourth passage virus has been sequenced and maintains a nucleotide sequence identical to that of the original clinical strain from the United States. These deposits make this virus strain 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 and enable a better understanding of the transmissibility and pathogenesis of this novel emerging virus.

Acknowledgments

We thank Mavanur R. Suresh for providing plasmid pBM302, which expresses the SARS-CoV nucleocapsid protein.

The reagent described is available through the Biodefense and Emerging Infections Research Resources Repository, National Institutes of Allergy and Infectious Diseases, National Institutes of Health: SARS-related coronavirus 2, isolate USA-WA1/2020, NR-52281.

This study was supported by grants from the National Institute on Aging and the National Institutes of Allergy and Infectious Diseases of the National Institutes of Health (U19AI100625 and R00AG049092 to V.D.M., R24AI120942 to S.C.W., and AI99107 and AI114657 to S.M.); a STARs Award provided by the University of Texas System to V.D.M.; the Institute for Human Infections and Immunity at the University of Texas Medical Branch (S.M.); and trainee funding provided by the McLaughlin Fellowship Fund at the University of Texas Medical Branch.

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Biomédica 2020;40(Supl.2):148-58
doi: https://doi.org/10.7705/biomedica.5834

Artículo original

Aislamiento y caracterización de una cepa temprana de SARS-CoV-2 durante la epidemia de 2020 en Medellín, Colombia

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Introducción. El nuevo coronavirus causante de un brote de enfermedad respiratoria aguda en China en diciembre de 2019 se identificó como SARS-CoV-2. La enfermedad, denominada COVID-19, fue declarada pandemia por la Organización Mundial de la Salud (OMS). El primer caso de COVID-19 en Colombia se reportó el 6 de marzo de 2020; en este estudio se caracterizó un aislamiento temprano del virus SARS-CoV-2 de una muestra recolectada en abril de 2020.
Objetivos. Describir y caracterizar una cepa temprana a partir de un aislamiento de SARS-CoV-2 durante la pandemia en Colombia.
Materiales y métodos. Se obtuvo una muestra de un paciente con COVID-19 confirmada por qRT-PCR; la muestra fue inoculada en diferentes líneas celulares hasta la aparición del efecto citopático. Para confirmar la presencia de SARS-CoV-2 en el cultivo, se utilizó la qRT-PCR a partir de los sobrenadantes, la inmunofluorescencia indirecta (IFI) en células Vero-E6, así como microscopía electrónica y secuenciación de nueva generación (*next-generation sequencing*).
Resultados. Se confirmó el aislamiento de SARS-CoV-2 en células Vero-E6 por la aparición del efecto citopático tres días después de la infección, así como mediante la qRT-PCR y la IFI positiva con suero de paciente convaleciente positivo para SARS-CoV-2. Además, en las imágenes de microscopía electrónica de trasmisión y de barrido de células infectadas se observaron estructuras compatibles con viriones de SARS-CoV-2. Por último, se obtuvo la secuencia completa del genoma, lo que permitió clasificar el aislamiento como linaje B.1.5.
Conclusiones. La evidencia presentada en este artículo permite confirmar el primer aislamiento de SARS-CoV-2 en Colombia. Además, muestra que esta cepa se comporta en cultivo celular de manera similar a lo reportado en la literatura para otros aislamientos y que su composición genética está acorde con la variante predominante en el mundo. Finalmente, se resalta la importancia que tiene el aislamiento viral para la detección de anticuerpos, para la caracterización genotípica y fenotípica de la cepa y para probar compuestos con potencial antiviral.

Palabras clave: infecciones por coronavirus; síndrome respiratorio agudo grave; virus del SARS; secuenciación de nucleótidos de alto rendimiento; microscopia electrónica; técnica indirecta del anticuerpo fluorescente.

Isolation and characterization of an early SARS-CoV-2 isolate from the 2020 epidemic in Medellín, Colombia

Introduction: SARS-CoV-2 has been identified as the new coronavirus causing an outbreak of acute respiratory disease in China in December, 2019. This disease, currently named COVID-19, has been declared as a pandemic by the World Health Organization (WHO). The first case of COVID-19 in Colombia was reported on March 6, 2020. Here we characterize an early SARS-CoV-2 isolate from the pandemic recovered in April, 2020.
Objective: To describe the isolation and characterization of an early SARS-CoV-2 isolate from the epidemic in Colombia.
Materials and methods: A nasopharyngeal specimen from a COVID-19 positive patient was inoculated on different cell lines. To confirm the presence of SARS-CoV-2 on cultures we used qRT-PCR, indirect immunofluorescence assay, transmission and scanning electron microscopy, and next-generation sequencing.
Results: We determined the isolation of SARS-CoV-2 in Vero-E6 cells by the appearance of the cytopathic effect three days post-infection and confirmed it by the positive results in the qRT-PCR and the immunofluorescence with convalescent serum. Transmission and scanning electron microscopy images obtained from infected cells showed the presence of structures compatible with SARS-CoV-2. Finally, a complete genome sequence obtained by next-generation sequencing allowed classifying the isolate as B.1.5 lineage.

Recibido: 30/09/2020
Aceptado: 15/10/2020
Publicado: 19/10/2020

Citación:
Díaz FJ, Aguilar-Jiménez W, Flórez-Álvarez L, Valencia G, Laiton-Donato K, Franco-Muñoz C, *et al.* Aislamiento y caracterización de una cepa temprana de SARS-CoV-2 durante la epidemia de 2020 en Medellín, Colombia. Biomédica. 2020;40(Supl.2):148-58. https://doi.org/10.7705/biomedica.5834

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Contribución de los autores:
María T. Rugeles, Gladys Valencia y Francisco J. Díaz: reclutamiento del paciente y obtención de datos epidemiológicos
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Katherine Laiton-Donato, Carlos Franco-Muñoz, Diego Álvarez-Díaz y Marcela Mercado-Reyes: secuenciación de nueva generación
Francisco J. Díaz, Wbeimar Aguilar-Jiménez, Lizdany Flórez-Álvarez y María T. Rugeles: escritura del manuscrito
Todos los autores revisaron y aceptaron la versión final del manuscrito.

Financiación:
Universidad de Antioquia

Conflicto de intereses:
Los autores declaran que no tienen ningún conflicto de intereses relacionado con este trabajo.

Conclusion: The evidence presented in this article confirms the first isolation of SARS-CoV-2 in Colombia. In addition, it shows that this strain behaves in cell culture in a similar way to that reported in the literature for other isolates and that its genetic composition is consistent with the predominant variant in the world. Finally, points out the importance of viral isolation for the detection of neutralizing antibodies, for the genotypic and phenotypic characterization of the strain and for testing compounds with antiviral potential.

Keywords: Coronavirus infections; viral isolation severe acute respiratory syndrome; SARS virus; high-throughput nucleotide sequencing; microscopy, electron; fluorescent antibody technique, indirect.

En diciembre de 2019 en Wuhan, República Popular de China, se describieron los primeros casos de una enfermedad febril con neumonía grave que se presentaba con un espectro clínico variable y, en algunos casos, resultados fatales, principalmente en adultos mayores y en pacientes con ciertas comorbilidades (1,2). A principios de 2020 se aisló en muestras de lavado broncoalveolar un nuevo agente que se identificó como un betacoronavirus (2,3). Este virus, inicialmente denominado 2019-nCoV (*novel coronavirus 2019*), se conoce hoy como SARS-CoV-2 por su similitud genética y clínica con otro betacoronavirus, el SARS-CoV, causante de la epidemia de síndrome respiratorio agudo grave (*Severe Acute Respiratory Syndrome,* SARS) que se presentó entre 2002 y 2003 en Asia y que se diseminó de forma limitada a otros países (4).

La enfermedad causada por el SARS-CoV-2, ahora denominada COVID-19 (*Coronavirus Disease 2019*), se ha extendido por todo el mundo causando más de 25 millones de casos confirmados y más de 850.000 muertes en los primeros ocho meses, lo que la ha convertido en la mayor pandemia de los últimos tiempos (*Coronovairus resource center, Johns Hopkins University (JHU) Global*, https://coronavirus.jhu.edu/map.html).

El origen de los coronavirus que afectan a los humanos parece ser zoonótico; de hecho, los betacoronavirus humanos están filogenéticamente relacionados con los coronavirus de murciélagos y pueden transmitirse a los humanos directamente o por medio de un huésped intermediario; según se ha sugerido, este es el caso del SARS-CoV-2 (4,5).

Al ser un virus con genoma ARN, el SARS-CoV-2 tiene una alta tasa de evolución, lo que ha llevado a que durante los pocos meses de la pandemia se haya descrito un gran número de variables (6). Entre los cambios genéticos ocurridos en los primeros meses de 2020 se destaca la mutación D614G, un cambio de ácido aspártico por glicina en la proteína S (*spike*) que hace más eficiente la unión del virus a su receptor en células humanas y que hoy predomina en todos los continentes (7). El efecto de estas variantes en términos de complicaciones clínicas se desconoce, aunque se ha establecido una correlación positiva entre la variante G614 y la tasa de mortalidad (8).

El primer caso confirmado de COVID-19 en Colombia se reportó el 6 de marzo de 2020 (9). En este estudio se describe el primer aislamiento del virus SARS-CoV-2 en Colombia –en abril de 2020– a partir de una muestra de aspirado nasofaríngeo de un paciente de Medellín.

Descripción del caso

El aislamiento provino de una muestra de aspirado nasofaríngeo de uno de los primeros pacientes diagnosticados con COVID-19 en Colombia.

Se trataba de un hombre de 59 años residente en Medellín con antecedentes de hipertensión arterial, diabetes mellitus e hipercolesterolemia, todas ellas en tratamiento en el momento de la infección.

El paciente había viajado por el norte de España, Madrid y Portugal durante 17 días y regresó a Medellín el 12 de marzo de 2020. A su llegada se le ordenó permanecer en confinamiento domiciliario. Cuatro días más tarde, el 16 de marzo, tuvo los primeros síntomas que incluían cefalea, dolor dorsal alto, malestar en la faringe, tos leve y fiebre (38,2 °C). En los días siguientes, el dolor dorsal se extendió por la espalda y el paciente presentó somnolencia, pérdida del apetito y ageusia sin anosmia, y se le trató únicamente con acetaminofén.

El 19 de marzo se le tomó un aspirado nasofaríngeo, el cual resultó positivo en la prueba de qRT-PCR (protocolo del Charité, Berlín) (10). En los días siguientes a la toma de la muestra el paciente presentó astenia prolongada y malestar mal definido en la espalda, pero con tendencia a la mejoría. El 25 de marzo, aún con sintomatología leve, se tomó otra muestra de aspirado nasofaríngeo y una de sangre para el aislamiento viral y la determinación de los títulos de anticuerpos, respectivamente. El 14 de abril (después del aislamiento) se tomó una nueva muestra de sangre durante la fase convaleciente. En el último control clínico, realizado por teléfono el 19 de abril, el paciente informó estar completamente recuperado.

Materiales y métodos

Muestras

Las muestras de sangre y aspirado nasofaríngeo se tomaron en las fechas mencionadas. El paciente dio su consentimiento después de ser informado sobre el propósito de la investigación. Parte de la muestra del aspirado nasofaríngeo fue sometida a la prueba de qRT-PCR para SARS-CoV-2 y el resto se conservó a -80 °C para el aislamiento viral.

Aislamiento viral

El aislamiento viral se hizo en el laboratorio de nivel 3 de bioseguridad (BSL-3) de la Sede de Investigación Universitaria de la Universidad de Antioquia, siguiendo las prácticas y procedimientos recomendados (11).

Se emplearon las líneas celulares LLC-MK2, Vero-76 y Vero-E6. Las células se cultivaron en DMEM (*Dulbecco's Modified Eagle Medium*, Sigma-Aldrich, St. Louis, MO, USA) con suplemento de suero bovino fetal (SBF) (Gibco, Grand Island, NY, USA) al 10 % y penicilina-estreptomicina (Sigma-Aldrich, St. Louis, MO, USA) al 1 % en frascos de cultivo celular de 25 cm^2. Una vez alcanzada una confluencia del 80 % se procedió a inocular la monocapa con 80 µl del aspirado nasofaríngeo diluidos en 1 ml de medio DMEM. Las células se incubaron a 37 °C con 5 % de CO_2 durante 90 minutos agitando suavemente cada 15 minutos. Después de la incubación se retiró el inóculo y se reemplazó con 5 ml de medio DMEM con 2 % de SBF y 1 % de penicilina-estreptomicina. Los cultivos se inspeccionaron bajo el microscopio diariamente para detectar el efecto citopático. Para cada línea celular se incluyó un control sin infección para determinar la apariencia de las células en ausencia del efecto citopático.

Detección del SARS-CoV-2 mediante RT-PCR en tiempo real

Se extrajo ARN viral a partir de la muestra de aspirado nasofaríngeo del paciente y de los sobrenadantes del cultivo de células inoculadas usando el estuche comercial QIAamp Viral RNA Mini Kit™ (Qiagen, Hilden, Germany) según las instrucciones de la casa comercial.

La transcripción inversa y la posterior amplificación del genoma viral del SARS-CoV-2 en tiempo real (qRT-PCR) se hizo en un solo paso utilizando el estuche comercial qScript XLT 1-Step RT-qPCR Tough Mix™ (Quantabio Beverly, MA, USA) con los oligonucleótidos y sondas del protocolo CDC RT-PCR para el gen *N1* (IDT, Coralville, Iowa USA) (secuencias disponibles en https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html).

Inmunofluorescencia indirecta

Para confirmar el aislamiento viral se utilizó inmunofluorescencia indirecta (IFI) en placas sensibilizadas con células Vero-E6. A los cuatro días de cultivo se desprendieron mecánicamente las monocapas de células Vero-E6 inoculadas y no inoculadas y se suspendieron de nuevo en 3 ml de tampón fosfato salino (PBS) (Lonza, Rockland, ME, USA). Se agregaron 20 µl/pozo de la suspensión de células en láminas portaobjetos de 12 pozos (Thermo Scientific, Wilmington, DE, USA), se dejaron secar y luego se fijaron por inmersión en acetona pura durante 15 minutos.

A partir del suero del paciente se hicieron diluciones seriadas dobles desde 1:5 hasta 1:80 en PBS. Se agregaron 20 µl de cada dilución a las células fijadas en las láminas y se incubaron en cámara húmeda a 37 ºC durante 30 minutos. Después de la incubación las placas se lavaron dos veces con PBS durante 5 minutos con agitación lenta, se dejaron secar y en cada pozo se agregaron 20 µl de conjugado Anti-human IgG (Fc specific)-FITC antibody produced in goat™ (Sigma-Aldrich) en PBS. Las placas se incubaron en cámara húmeda a 37 °C durante 30 minutos protegidas de la luz, luego se lavaron dos veces con PBS y se montaron con anti-fade Fluosaver™ (Calbiochem) y lámina cubreobjetos. Las placas se visualizaron en un microscopio invertido de luz fluorescente Axio Vert.A1™ (Zeiss, Oberkochen, Alemania) con 400X.

Caracterización viral mediante microscopía electrónica

El estudio ultraestructural del virus se hizo mediante microscopía electrónica de transmisión y microscopía electrónica de barrido en el Centro de Microscopía Avanzada de la Universidad de Antioquia. Para la microscopía electrónica de transmisión se procesaron células Vero-E6 inoculadas con la muestra nasofaríngea después de la aparición del efecto citopático. Estas se fijaron con glutaraldehído al 2,5 % y posteriormente se fijaron con tetróxido de osmio al 1-2 %. A continuación, las células se lavaron en PBS dos veces y se trataron con alcoholes en concentraciones ascendentes (70, 95 y 100 %) para deshidratarlas. Después, las muestras se embebieron en resina epóxica y se sometieron a polimerización en cápsulas para obtener bloques de los cuales se sacaron cortes de 60 a 90 nanómetros con un ultramicrótomo. Estos se montaron en rejillas y se contrastaron inicialmente con acetato de uranilo y luego con citrato de plomo. Los cortes en las rejillas se observaron en un microscopio electrónico de transmisión Tecnai G2 F20™ (FEI Company, Hillsboro, OR, USA).

Para la microscopía electrónica de barrido las muestras deshidratadas se colocaron en un secador de punto crítico SPC SAMDRI-795™ (Tousimis, Rockville, MD, USA). Luego se fijaron en una cinta de grafito, se les realizó un recubrimiento delgado en oro y se analizaron en el microscopio electrónico de barrido JEOL JSM 6490 LV™ (JEOL; Peabody, MA, USA) en alto vacío. Se empleó el detector de electrones secundarios para evaluar la morfología y la topografía de las muestras.

Cuantificación de los títulos virales mediante ensayo en placa

El virus aislado se tituló mediante ensayo en placa de monocapas de células Vero-E6. Las células se cultivaron en medio DMEM con suplemento de 10 % de SBF a 37 °C y 5 % de CO_2. Para preparar las placas se sembraron células Vero-E6 en una densidad de 1 x 10^5 células/pozo en platos de 24 pozos con 500 µl de medio DMEM y suplemento de 2 % de SBF; las células se incubaron durante 24 horas a 37 °C con 5 % de CO_2 y después de la incubación se infectaron durante una hora con 200 µl/pozo de diluciones en base diez del aislamiento viral a 37 °C con 5 % de CO_2. Pasada una hora, se retiró el inóculo y se reemplazó con 1 ml de medio semisólido (DMEM con 2 % de SFB más 1,5 % de carboximetilcelulosa). Las células se incubaron durante cuatro días a 37 °C con 5 % de CO_2.

Pasados cuatro días se retiró el medio semisólido y se lavaron las células dos veces con PBS. Posteriormente, se hicieron la tinción y fijación con 500 µl por pozo de una solución de formaldehído al 4 % y cristal violeta al 1 % durante 30 minutos a temperatura ambiente. Por último, las células se lavaron dos veces con PBS.

Para determinar el título viral, se hizo el recuento de las placas. El promedio de dos réplicas se multiplicó por el inverso de la dilución y el volumen del inóculo para obtener el número de unidades formadoras de placa por ml (UFP/ml). Para establecer el título del aislamiento en UFP/ml se hicieron tres experimentos independientes.

Secuenciación de nueva generación

Para secuenciar el genoma se utilizó el ARN extraído del sobrenadante del cultivo de células con efecto citopático y resultado positivo en la qRT-PCR. La preparación y secuenciación de la librería se hizo con tecnologías de nanopore siguiendo el protocolo ARTIC (12). Se obtuvo un conjunto de amplicones de ~400 pb de todo el genoma del SARS-CoV-2 con los cebadores nCoV-2019/V3 (13).

Los amplicones se mezclaron, se cuantificaron y se etiquetaron con el estuche Native Barcoding Kit EXPNBD104™ (Oxford Nanopore Technologies, Oxford, UK) y se combinaron en una cantidad equimolar. Se prepararon librerías genómicas con el estuche de ligadura 1D SQK-LSK109™ (Oxford Nanopore Technologies) y se secuenciaron usando una celda de flujo FLO-MIN106-R9.4™ y el instrumento MinION™ (Oxford Nanopore Technologies).

Las bases nitrogenadas se identificaron usando Guppy, versión 3.2.2™ (Oxford Nanopore Technologies). Las lecturas procesadas se alinearon con el genoma de referencia del SARS-CoV-2 (GenBank NC_045512.2) utilizando el algoritmo BWA-MEM (14) y el BBMap (https://www.osti.gov/biblio/1241166-bbmap-fast-accurate-splice-aware-aligner) para generar la secuencia de consenso. Por último, el linaje de la secuencia se clasificó usando PANGOLIN (Phylogenetic Assignment of Named Global Outbreak LINeages) (15).

Resultados

Aislamiento viral

Antes del aislamiento se hizo la qRT-PCR a la muestra tomada el 25 de marzo, la cual resultó positiva con un ciclo umbral de 19, lo que demostró que el paciente seguía excretando gran cantidad de virus. A los tres días de la infección,

las monocapas de células control eran confluentes, en tanto que las monocapas inoculadas con la muestra mostraban un efecto citopático significativo (figura 1A-B). Se tomaron muestras de sobrenadante y se incubó el cultivo hasta el día siguiente. Al cuarto día de la infección se observó un desprendimiento del 65 % de la monocapa y se recolectaron las células para la IFI.

Con el sobrenadante recolectado a los tres días de la infección se hizo la qRT-PCR con oligos específicos para el gen *N* del SARS-CoV-2, con resultado positivo y un ciclo umbral de 12 ciclos. En la IFI realizada con el primer suero del paciente se observó una fluorescencia de baja intensidad (figura 1 C y D). Sin embargo, cuando se repitió con el suero del paciente ya convaleciente se obtuvo una fluorescencia con un patrón periférico bien definido (figura 1E).

Caracterización viral mediante microscopia electrónica

Para comprender mejor la morfología del aislamiento de SARS-CoV-2 se utilizó microscopía electrónica en las partículas virales. En la muestra de células Vero-E6 con efecto citopático observadas con microscopía electrónica de transmisión, estas presentaron tamaños entre los 86 y los 180 nm de diámetro (figura 2A y 2B). Aunque no se evidenció en ellas la típica apariencia de corona, **s**í se observaron algunas estructuras compatibles con espículas (figura 2A). En cortes finos las espículas virales no suelen preservarse (Dr. Vsevolod Popov, UTMB, Galveston TX, USA, comunicación personal). La forma y el tamaño de los viriones se confirmaron mediante microscopía electrónica de barrido. Las partículas virales se observan redondas y llenas, algunas de ellas adheridas unas a otras (figura 2C). También se observaron imágenes sugestivas de viriones en proceso de gemación y partículas elongadas de identidad desconocida (figura 2D).

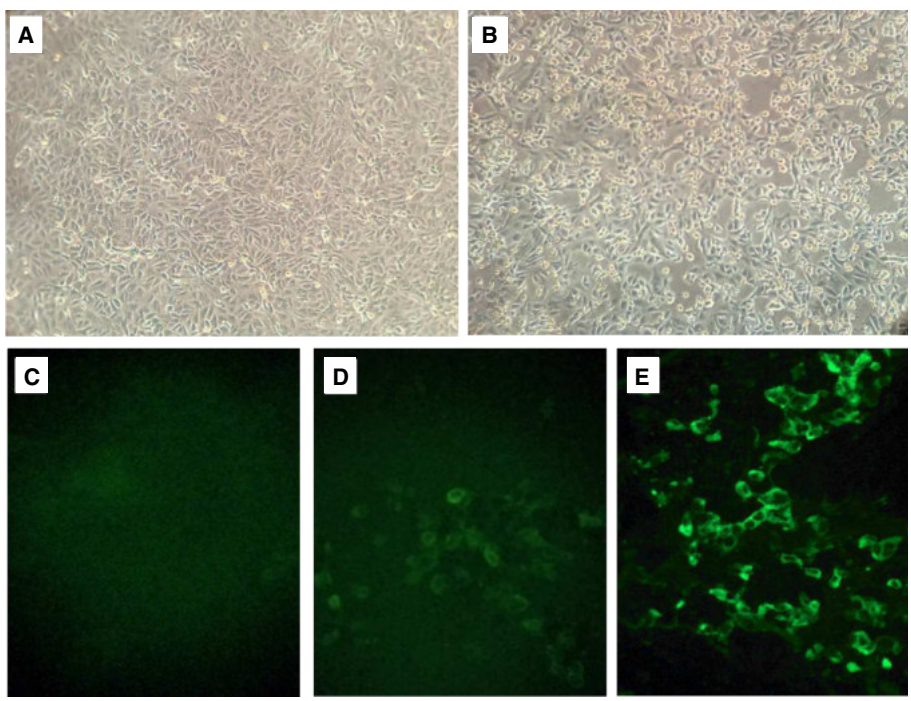

Figura 1. Identificación de un aislamiento colombiano de SARS-CoV-2. A. y B. Monocapa de células Vero-E6 sin infectar e infectadas; se observa el efecto citopático del SARS-CoV-2 en monocapas de células Vero-E6 a tres días de la inoculación. C., D. y E. Fotografías de placas de IFI preparadas con el suero del paciente en células no infectadas, suero de la etapa aguda en células infectadas y suero de convaleciente en células infectadas

Cuantificación de los títulos virales mediante ensayo en placa

Se obtuvieron títulos de 3,4 ± 0,7 x 10^6 UFP/ml a los 4 días después de la infección en el ensayo en placas con células Vero E6. En estas monocapas se evidenció que el SARS-CoV-2 generó dos tamaños de placa como se observa en la figura 3.

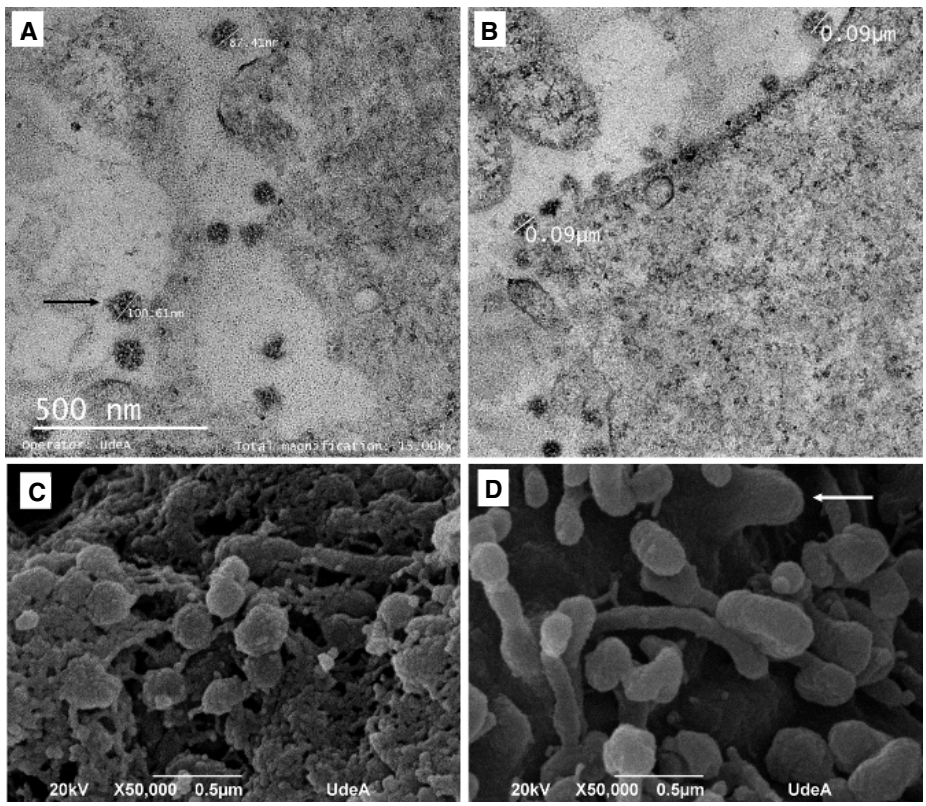

Figura 2. Microscopia electrónica de células Vero-E6 infectadas. A. y B. Fotografías obtenidas por microscopía electrónica de transmisión de partículas virales con tamaño variable entre 87 y 180 nm de diámetro; en A. la flecha señala una estructura compatible con espículas de viriones de SARS-CoV-2. C. y D. Fotografías obtenidas por microscopía electrónica de barrido; se observan estructuras con morfología y tamaño compatibles con de viriones de SARS-CoV-2. En D. también se observa un virión en proceso de gemación (flecha).

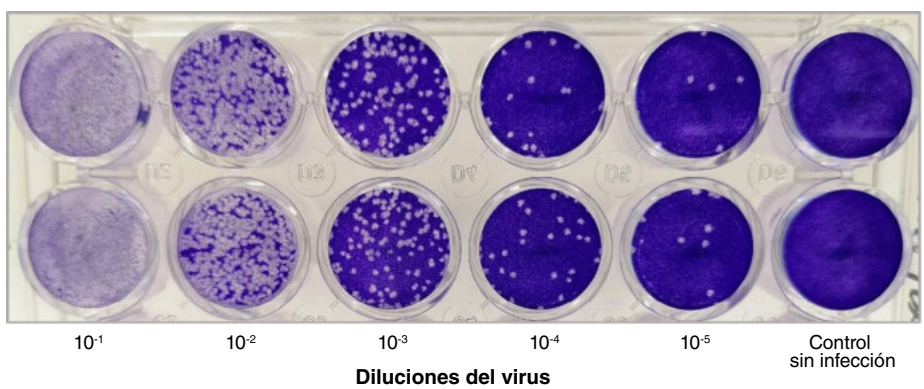

Figura 3. Placas con SARS-CoV-2 en monocapa de células Vero-E6. Esta imagen es representativa de la preparación de placas con SARS-CoV-2 en monocapas de células Vero-E6. Se fijaron y se hizo su tinción con una solución de 4 % de formaldehído y 1 % de cristal violeta a los cuatro días de la infección.

Caracterización genómica del aislamiento viral

El análisis de la secuencia genómica obtenida evidenció una profundidad de 4.600 x o más y una cobertura del 99,5 %. Cuando se comparó con la secuencia de referencia NC_045512.2 (Wuhan-HU1-2019), se observaron cuatro diferencias en las posiciones 241, 3.037, 14.408 y 23.403 del genoma. Estas dos últimas son sustituciones no sinónimas y corresponden a las mutaciones P323L en la polimerasa viral y D614G en la proteína S del SARS-CoV-2. El genoma se clasificó como linaje B.1.5 con un valor de *bootstrap* de 92 y un valor de SH-aLTR de 100, resultado que coincide con linajes previamente descritos en el país (16). La secuencia fue depositada en la base de datos GISAID (www.gisaid.org) con el nombre hCoV-19/Colombia/ANT-UdeA-200325-01/2020 y código de acceso EPI ISL 536399.

Discusión

El objetivo de este estudio era aislar una cepa de SARS-CoV-2 a partir de una muestra de un paciente con COVID-19. Los resultados obtenidos mediante las pruebas qRT-PCR e IFI, y las imágenes de microscopía electrónica de transmisión y de barrido y la secuenciación de nueva generación en el cultivo celular inoculado con la muestra nasofaríngea del paciente estudiado permitieron concluir más allá de cualquier duda que se logró aislar una cepa de SARS-CoV-2.

Este es el primer aislamiento del virus en Colombia. Se evidenció que la cepa se comport**ó** *in vitro* de manera similar a lo descrito para otras cepas de SARS-CoV-2: el efecto citopático apareció en el tercer día con encogimiento y desprendimiento celular y un patrón mixto de placas grandes y pequeñas en el ensayo en placas con **c**élulas Vero-E6 (17,18).

El aislamiento se logró a pesar de que la muestra fue tomada menos de 13 días después de la infección y a nueve días del inicio de los síntomas. Se observ**ó** un ciclo umbral de 19 en la rRT-PCR y se alcanzó un crecimiento viral solo tres días después de la inoculación del cultivo. Esto indica una replicación eficiente del virus, con una alta excreción de partículas virales infecciosas, por lo menos, en algunos pacientes sintomáticos con COVID-19. Este resultado alerta sobre la inconveniencia de reducir el período de aislamiento de los pacientes infectados, que originalmente era de 14 días pero luego se redujo a diez (19).

La secuenciación de nueva generación permitió la identificación de cuatro sustituciones en las posiciones 241, 3.037, 14.408 y 23.403 del genoma viral y del linaje genético B.1.5". La última de dichas mutaciones representa la sustitución D614G a nivel de aminoácidos en la proteína S o espícula del virión. Dicha proteína incluye el dominio de unión al receptor y, por consiguiente, determina el tropismo tisular del virus; además, constituye el principal antígeno viral, dado que los anticuerpos neutralizantes se unen específicamente a dicha proteína.

En ensayos con pseudovirus se ha demostrado que las variantes G614 generan títulos virales mayores comparados con las D614 en diferentes líneas celulares (7). Esta mayor capacidad infecciosa se ha correlacionado con un aumento de la estabilidad y la tasa de incorporación de la proteína S en la membrana de los viriones (20). Se ha demostrado, además, que la mutación D614G aumenta la capacidad replicativa *in vitro* del SARS-CoV-2 en células epiteliales de pulmón y en cultivos primarios de tejido de

las vías respiratorias (21). Aunque en modelos animales no se observaron diferencias significativas en los signos clínicos como la pérdida de peso, sí se ha reportado que las variantes G614 producen mayores cargas virales en los lavados traqueales y nasales, pero no en los pulmonares (21). Por otra parte, se ha reportado que la infección con variantes D614 genera una mejor actividad de anticuerpos neutralizantes contra G614, lo que sugiere que esta mutación no reduciría la inmunidad conferida por la primera de estas variantes en infectados o vacunados contra la COVID-19 (21).

Con base en esta información se puede concluir que, aunque no hay evidencia de que las variantes G614 sean más letales, sí es cierto que la transmisión es más eficiente, lo que implica una ventaja evolutiva que podría explicar la rápida expansión de esta mutación. En efecto, los virus con la sustitución D614G incrementaron su frecuencia durante los primeros meses de la pandemia de COVID-19, incluso en regiones donde la D614 era dominante en un comienzo; esta transición se ha dado de manera asincrónica y en diferentes regiones alrededor del mundo: primero en Europa y Norteamérica y luego en Asia y Oceanía (7).

Como método diagnóstico, el aislamiento en cultivo celular se ha ido reemplazando por métodos más sensibles como la amplificación de genomas virales, incluida la prueba de qRT-PCR, que constituye el estándar diagnóstico para la infección por SARS-CoV-2; otras ventajas de los métodos moleculares son un menor tiempo de ejecución y un menor riesgo biológico para el laboratorista.

Sin embargo, en este estudio se pudo comprobar la utilidad del aislamiento viral para varios fines específicos: primero, para determinar el estado contagioso del paciente infectado; se ha observado que las muestras positivas para SARS-CoV-2 en la qRT-PCR con ciclos umbrales altos no se asocian con una excreción viable de virus, es decir, la qRT-PCR no permite diferenciar entre pacientes infectados y pacientes infecciosos (22).

Segundo, la disponibilidad del virus vivo permite la producción de antígenos virales utilizables en las pruebas de detección de anticuerpos por inmunofluorescencia indirecta (figuras 1D y 1E); además, dada la nítida formación de dichas placas en monocapas de células Vero-E6, la detección de anticuerpos neutralizantes con el método de reducción de placas cobrará cada vez más importancia a medida que se introduzcan las esperadas vacunas contra la COVID-19 (figura 3).

En tercer lugar, el aislamiento viral facilita la secuenciación del ARN viral, como lo demostró la cobertura del 99,5 % y la gran profundidad del genoma del virus aislado; a menudo la secuenciación directa a partir de muestras no permite obtener el genoma completo, especialmente en aquellas con baja carga viral.

Por último, la disponibilidad del virus vivo permite probar sustancias con posible acción antiviral, incluidos nuevos fármacos antivirales, antivirales de segundo uso, productos naturales y compuestos químicos o procesos físicos de desinfección para la limpieza de superficies, objetos o sustancias que puedan albergar virus infecciosos, procesos que hoy se están haciendo en nuestros laboratorios.

Agradecimientos

Al paciente cuyas muestras utilizamos por su decidida colaboración con este proyecto. Al Laboratorio Departamental de Salud Pública de Antioquia por proporcionar los datos epidemiológicos. A Gustavo Gámez, profesor de la Universidad de Antioquia, por su análisis genómico preliminar. Al personal del Centro de Microscopía Avanzada de la Sede de Investigación Universitaria. A los doctores Vsevolod Popov y Patricia Aguilar de University of Texas Medical Branch, UTMB, Galveston, TX, USA, por su ayuda en la interpretación de las imágenes de microscopía electrónica.

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Received: 13 March 2020 | Accepted: 26 March 2020

DOI: 10.1002/jmv.25797

RESEARCH ARTICLE

JOURNAL OF MEDICAL VIROLOGY WILEY

Phylogenetic analysis of the first four SARS-CoV-2 cases in Chile

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Abstract

The current pandemic caused by the new coronavirus is a worldwide public health concern. To aboard this emergency, and like never before, scientific groups around the world have been working in a fast and coordinated way to get the maximum of information about this virus when it has been almost 3 months since the first cases were detected in Wuhan province in China. The complete genome sequences of around 450 isolates are available, and studies about similarities and differences among them and with the close related viruses that caused similar epidemics in this century. In this work, we studied the complete genome of the first four cases of the new coronavirus disease in Chile, from patients who traveled to Europe and Southeast Asia. Our findings reveal at least two different viral variants entries to Chilean territory, coming from Europe and Asia. We also sub-classified the isolates into variants according to punctual mutations in the genome. Our work contributes to global information about transmission dynamics and the importance to take control measures to stop the spread of the infection.

KEYWORDS

COVID-19, phylogeny, SARS-CoV-2

1 | INTRODUCTION

Mankind is facing a new viral outbreak that originated in the Wuhan province, Hubei region in China. The new virus, a coronavirus named SARS-CoV-2, was reported in December 2019. Since then, it has reached over 110 countries and territories with more than 125 000 reported cases at the time of this report.[1] This new coronavirus disease (COVID-19) has caused more than 3100 deaths, mainly in continental China and mostly on elderly people, who are affected by fever and serious respiratory diseases like pneumonia.[2-4]

SARS-CoV-2 has a single-stranded RNA genome and its length is similar to other related coronaviruses, with an extension near 29 890 bp (GenBank NC_045512.2). The most related genomes available in public databases were bat-SL-CoVZC45 (GenBank MG772933) with an 87.99% sequence identity and bat-SL-CoVZXC21 (GenBank MG772934) with an 87.23% sequence identity,[5] followed by the human viruses SARS-CoV-Tor2 (GenBank NC_004718) and MERS-CoV (GenBank NC_019843) with a 79.0% and 51.8% of nucleotide identity, respectively.[6]

The genome organization of SARS-CoV-2 was shown to be similar of the related bats and human coronavirus. The open reading frames (ORFs) from 5′ to 3′ is as follows: 5′ UTR; ORF1ab with 16 nonstructural proteins (nsp) 1 to 16 including RNA polymerase RNA-dependent nsp12, Helicase nsp13 and 3′-to-5′ exonuclease nsp14, S surface spike protein, E envelope protein, M membrane protein, and N nucleocapsid protein. There are also at least six predicted ORFs as hypothetical proteins with no associated function.[4,6,7]

Near 286 complete genomes of SARS-CoV-2 and related viruses, has been submitted to the GISAID database (www.gisaid.org/CoV2020) collecting genetic information of the outbreak worldwide. The genomic sequences of all SARS-CoV-2 viruses isolated from patients share a sequence identity about a 99.9%,[7] suggesting a recently zoonotic infection, originated most probably from bats.[5,6,8]

As the information appears daily, new insights and concepts are being adopted and implemented. Recently Tang et al[9] and GISAD database in the SARS-CoV-2 portal defined three subtypes: S, G, and V, according to nucleotide variants that produce amino acid changes. These changes are located in ORF8 L84S; S (spike protein) D614G and nsp3 G251V, in the nucleotide position 28144, 23403, and 3471, respectively, for S, G, and V, according to the reference sequence NC_045512.2.

Chile was the fourth country in South America after Brazil, Ecuador, and Argentina to report COVID-19 in the region. In this report, we present the sequence analysis for the first four complete genomes for SARS-CoV-2 isolates on Chilean patients. Also, a phylogenetic study was performed with worldwide SARS-CoV-2 sequences and the full genomes from Chilean isolates, to identify their genetic similarity.

2 | MATERIALS AND METHODS

2.1 | Epidemiological information

The four cases presented in this report have contracted the infection abroad, either in Southeast Asia or Europe. The first two cases (20-18918, 20-19303) correspond to a couple in their early thirties, who traveled from Chile to Barcelona, Spain, where they stayed between January 27th and January 30th. On January 31th they traveled to Singapore, between February 4th to February 12th they were in Indonesia, then they visited Malaysia on February 13th, and the Maldives on February 15th. Between February 21st and 24th they stayed in Madrid, from where they traveled back to Chile arriving in Santiago on February 25th. The man showed symptoms first and was diagnosed as the first SARS-CoV-2 case in Chile, followed by his spouse 1 day later. That same day, a third case (20-19305) was confirmed. A 56-year-old woman who visited London between February 22nd and 23rd, Venice (February 23rd -25th), London (February 25th-28th), Madrid (February 28th to March 3rd) when she returned to Santiago. On the next day (March 5th) the fourth case (20-19731) was reported, a 40-year-old woman, who traveled to Milan between February 25th and 29th, traveling back to Chile.

2.2 | Sample types, RNA extraction, and virus detection

Chilean law by the Supreme Decree 7/2019 mandates notification of communicable diseases and their surveillance. All cases showed mild symptoms, and throat swab specimens were collected. A volume of 140 µL of each sample was used for viral RNA extraction with QIAamp Viral Mini Kit (Cat. No. 52926; Qiagen) in a QIACube extractor. All suspicious cases were confirmed by real-time reverse transcription-polymerase chain reaction (RT-PCR), using specific probe and primers, synthesized and purified in our facilities, targeting the RNA-dependent RNA polymerase (RdRp) region of SARS-CoV-2, according to the guidelines suggested by the World Health Organization.[10] SuperScript III One-Step RT-PCR Platinum Taq DNA Polymerase (Cat. No. 12574026; Invitrogen) was used for real-time RT-PCR. Running method 55°C for 10 minutes, followed by 94°C for 2 minutes, and 45 cycles at 94°C for 15 seconds and 58°C for 30 seconds. Ct's under 35 were considered as positive cases.

2.3 | Full viral genome amplification

From total RNA extraction we performed the first amplification round using SuperScript III One-Step RT-PCR Platinum Taq DNA Polymerase (Invitrogen) and six pair of specific primers to obtain six complementary DNA fragments around 5 Kbp each, followed by a second amplification round with 24 specific primers (Table S1) to generate two fragments from each first round products, each subfragment (a total of 12) are around 2.3 to 2.7 Kbp.

2.4 | Library generation and sequencing

The 12 DNA fragments from full genome amplification were pooled, and libraries were prepared with the Nextera XT Library Prep Kit (Illumina, San Diego, CA), purified with Agencourt AMPure XP beads (Beckman Coulter, Brea, CA) and quantified by Victor Nivo Fluorometer (PerkinElmer) using Quant-it dsDNA HS Assay kit (Invitrogen). The resulting DNA libraries were sequenced on MiSeq (Illumina) using a 300-cycle reagent kit. About 0.3 GB of data was obtained for each sample.

2.5 | Phylogenetic analysis

The sequencing quality was analyzed with software Fastqc v0.11.8 and then, the reads were filtered and trimmed using BBDuk software considering a minimum read length of 36 bases and quality more than equal to 10. SARS-CoV-2 assembly was performed with IRMA v0.9.3 using as reference NCBI sequence ID NC_045512.2. Sequence alignment was performed with MAFFT. The phylogenetic tree was built with IQ-TREE v1.6.12 considering a bootstrap of 1000. We consider 218 full complete genome sequences available in the GISAID platform plus the full genome sequences from the first four Chilean cases.

3 | RESULTS

The first four cases in Chilean territory were reported between March 3rd and 5th. All of these persons reported travel to places

TABLE 1 Nucleotide substitutions for Chilean virus isolates compared to the reference strain NC_045512.2

SARS-CoV-2 sample	Nucleotide position	Base change	Open reading frame	Amino acid substitution
20-18918	8782	C→T	ORF1ab-transmembrane domain 2 (TM2)	Silent (S)
	17470	C→T	ORF1ab-nsp13-helicase (HEL)	Silent (L)
	18907	G→T	ORF1ab-nsp14-3′-to-5′ exonuclease	V290F
	26088	C→T	ORF3a	Silent (I)
	28144	T→C	ORF8	L84S
	28580	G→T	N–Nucleocapsid phosphoprotein	D103Y
20-19303	8782	C→T	ORF1ab-transmembrane domain 2 (TM2)	Silent (S)
	17470	C→T	ORF1ab-nsp13-helicase (HEL)	Silent (L)
	18907	G→K	ORF1ab-nsp14-3′-to-5′ exonuclease	No change/V290F
	26088	C→T	ORF3a	Silent (I)
	28144	T→C	ORF8	L84S
	28580	G→T	N-Nucleocapsid phosphoprotein	D103Y
20-19305	1884	C→Y	ORF1ab–nsp2	Silent/A540V
	8782	C→T	ORF1ab-transmembrane domain 2 (TM2)	Silent (S)
	9477	T→A	ORF1ab-transmembrane domain 2 (TM2)	F308Y
	14807	C→T	ORF1ab-nsp12-RNA-dependent RNA polymerase	Silent (Y)
	25979	G→T	ORF3a	G193V
	28144	T→C	ORF8	L84S
	28657	C→T	N–Nucleocapsid phosphoprotein	Silent (D)
	28863	C→T	N–Nucleocapsid phosphoprotein	S197L
20-19371	241	C→T	5′ UTR	…
	3037	C→T	ORF1ab–nsp3–papain-like proteinase	Silent (F)
	3393	C→T	ORF1ab–nsp3–papain-like proteinase	A225V
	14408	C→T	ORF1ab-nsp12-RNA-dependent RNA polymerase	P323L
	23403	A→G	S-Surface Glycoprotein (Spike)	D614G
	28881	G→A	N–Nucleocapsid phosphoprotein	R203K
	28882	G→A	N–Nucleocapsid phosphoprotein	R203K
	28883	G→A	N–Nucleocapsid phosphoprotein	G204R

where the presence of the virus was confirmed and with an increasing number of cases.

We identified the SNPs that generates amino acid changes in all four Chilean genomes (Table 1). For the first and second samples (couple) the sequences are identical and the SNPs generates non-synonymous mutations in ORF1ab-nsp14-3′-to-5′ exonuclease (V290F), ORF8 (L84S) and N-nucleocapsid phosphoprotein (D103Y). The third Chilean case present mutations in the ORF1ab-transmembrane domain 2 TM2 (F308Y), ORF3a (G193V), ORF8 (L84S), and N-nucleocapsid phosphoprotein (S197L). The fourth case present mutations in ORF1ab- nsp3-papain-like proteinase (A225V), ORF1ab-nsp12-RNA-dependent RNA polymerase (P323L), S-surface spike glycoprotein (D614G), and in the N-nucleocapsid phosphoprotein twice (R203K, G204R).

According to prevalent SNPs, all genomes have been classified by amino acid changes in specific ORFs. For the Chilean strains, the first three cases (20-18918, 20-19303, 20-19305) are classified as "S" type, meanwhile, the fourth case (20-19731) is a "G" type, according to nucleotide substitutions in the positions 28 144 and 23 403, respectively.

A maximum-likelihood phylogeny tree was constructed using 218 complete genome sequences plus the four Chilean cases. Our first two samples, the married couple, mapped together (100% nucleotide identity) and with strains from Wuhan, China and Taiwan. The third sample groups in a well-defined clade with Spanish isolates. The fourth Chilean strain, groups in a European clade with samples from Switzerland, Netherlands, and Germany. In this same clade, we can identify one of the Brazilian and Mexican isolates, representing isolates from Latin America. In addition, the complete genomes were colored according to the variant groups, defined by specific mutations (Figure 1).

4 | DISCUSSION

In this early stage of the epidemic, sharing data and information is crucial and the efforts of the scientist worldwide are admirable. After a few weeks since the outbreak started in Wuhan province, the full genome sequence of SARS-CoV-2 was available, and this information paved the way for the development of better detection protocols

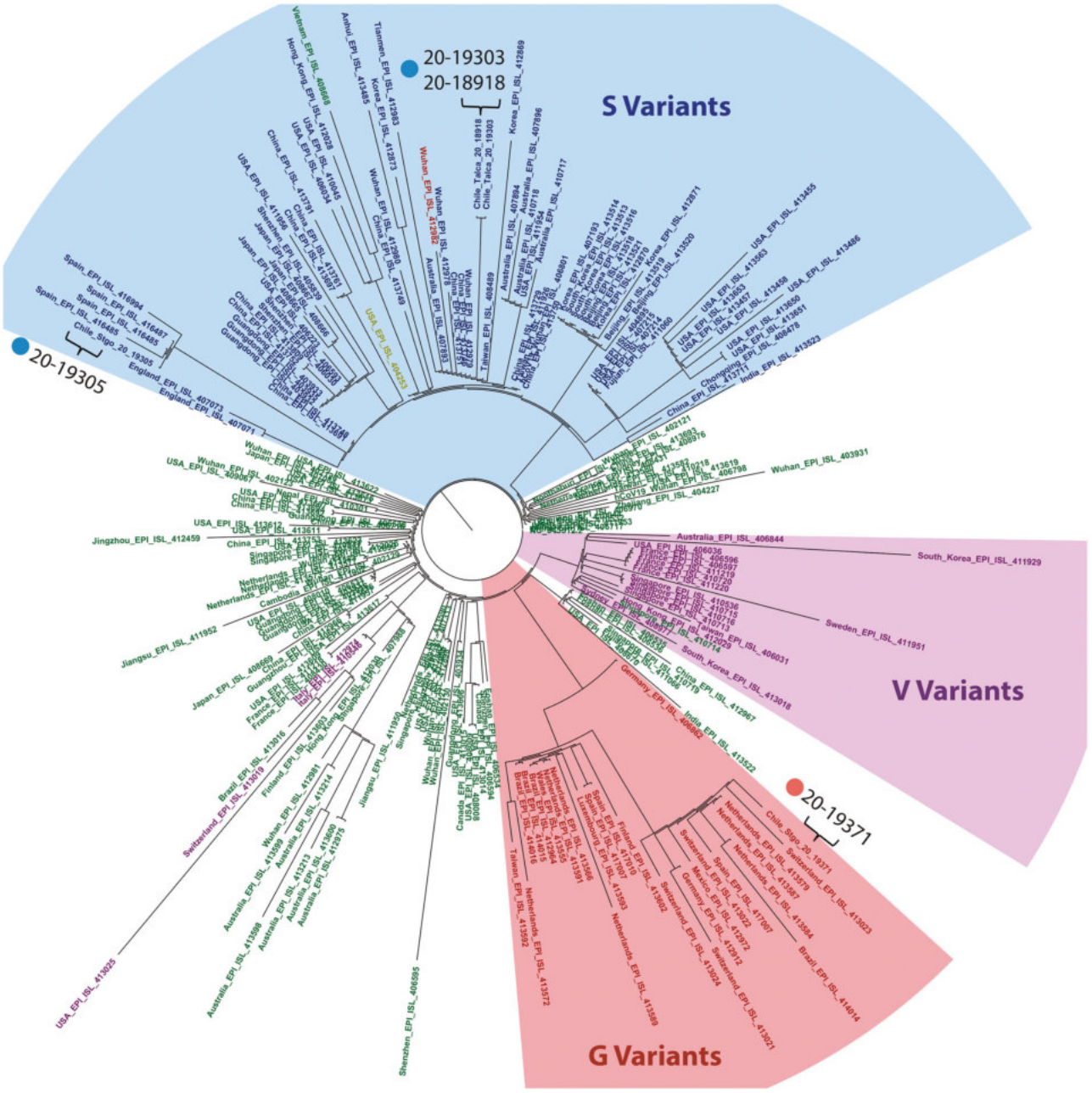

FIGURE 1 Phylogenetic tree with Maximum Composite Likelihood distance, representing 222 complete genomes including the four Chilean isolates. The name of the isolates were colored according to the variants as follows: S (blue), G (red), V (purple), unclassified variants (green), and the main clades were highlighted. Chilean strains are marked over the cladogram in the S and G variant clades

techniques, antiviral strategies, and phylogeny studies, among others scientific challenges. Here we report the first four cases of COVID-19 and the complete genome sequencing for these strains. We developed an RT-PCR based strategy to amplify the whole genome in two steps, followed by the library construction and further NGS using Illumina MiSeq. In less than 5 days since we detected the first case, the whole genomes for the first four cases were assembled. We implemented in early January the complete detection system by real-time RT-PCR for this new virus in the Public Health Institute of Chile, according to international guidelines.

The phylogenetic analysis plus the travel information of each patient, allows us to infer about the viral entries to Chilean territory. We detected two different viral variants entries to Chile, the S and G. For S variant, the viral genome distribution of Chilean isolates allows to associate these in two different clades, one related to the Wuhan province in China and Taiwan, and a second clade related with Spanish isolates, coincident with the patient's travel record. COVID-19 cases in Spain started to be reported since February 1st with a very low number of cases for about a month, where the reported cases may be underestimated, and after a month the infected

people started to raise over a hundred patients.[11,12] In the case of the G variant entry, the infected patient visited Europe and the complete sequence genome for this case, groups with isolates from the Netherlands, Switzerland, and Spain among other European countries (Figure 1). At the date of this report, we have detected more than 30 positive cases, mainly from Chilean travelers returning to the country and local transmission between their closest relatives.

At the beginning of the pandemic (until mid-February), when 99% of the cases were focused in continental China, the death toll was about 2%. As the virus is spread by travelers, the number of cases and death occurrence has increased in other territories like South Korea, Iran, and Italy. In this last country the death toll up to date reaches the 6.2%, this number is still far from statistics of other related human coronavirus epidemics, like SARS (9.5%) and MERS (34.4%).[13] Until now, there is no enough evidence to relate specific mutation in the viral genome to a higher number of infected patients or even death, the main number of fatalities is still related to the elderly population.

In conclusion, our work presents the complete genome analysis for the first cases of COVID-19 in Chile, detecting at least two different viral variants entries to Chilean territory. This information contributes to monitoring the spread of the infection and the surveillance for eventual recombination or genome mutations that the diversity of host, countries, weather conditions and other selective pressures that this new coronavirus could face. The globalization, increment of worldwide travelers and the high contagious rate of this virus require severe control measures to control infection dissemination.

ACKNOWLEDGMENTS

The authors are thankful to María Ibañez and Jorge Lobos for their valuable technical assistance.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

CAE participated in conceptualization, study design, interpreting the data analysis, methodology design, visualization, and wrote the whole manuscript. PB participated in methodology design and experimental assays, TP and TJ participated in data analysis and bioinformatics support. LJ, AL, and BG contributed to genome sequencing. AA, AW, LG, TC, and BP, participate in sample processing and real-time RT-PCR assays. US and FR participated in the critical review of the content. FJ contributed to the conceptualization, study design, supervision, critical review of the content, and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Castillo AE, Parra B, Tapia P, et al. Phylogenetic analysis of the first four SARS-CoV-2 cases in Chile. *J Med Virol*. 2020;92:1562–1566. https://doi.org/10.1002/jmv.25797

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LETTER TO THE EDITOR

Isolation and Full-Length Genome Characterization of SARS-CoV-2 from COVID-19 Cases in Northern Italy

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Danilo Licastro and Sreejith Rajasekharan contributed equally to this work and are named in alphabetical order.

KEYWORDS COVID-19, Italy, SARS-CoV-2

In December 2019, the novel coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in the city of Wuhan in Hubei province, People's Republic of China, as the etiologic agent of coronavirus disease 2019 (COVID-19), which has hence spread worldwide causing a global pandemic (1–3). The epidemic has been growing exponentially in Italy for the last month, affecting over 60,000 individuals so far and with a heavy mortality burden. Italy is only anticipating what will be the trend in the whole of Europe and elsewhere. At the beginning of March 2020, the first nasopharyngeal swabs positive for SARS-CoV-2 started to be detected in the Northern Eastern Region of Friuli-Venezia Giulia. These identifications followed the expansion of the two clusters in Lombardy and Veneto that emerged in the previous weeks in northern Italy (4). Swab contents were seeded on Vero E6 cells and monitored for cytopathic effect and by an RT-PCR protocol using primers for the N region (5). Cell culture supernatants from passage 1 (P1) of four isolates were collected, and RNA was extracted with QIAamp viral RNA minikit (Qiagen) and quantified with an *in vitro*-transcribed RNA standard (S. Rajasekharan and A. Marcello, unpublished data). The quantity and quality of the RNA were assessed using Qubit 2.0 fluorometer (Thermo Fisher Scientific) and Agilent 2100 Bioanalyzer (Agilent Technologies). For each sample, 100 ng of total RNA was processed using Zymo-Seq RiboFree ribosomal depletion library preparation kit (Zymo Research). All the obtained libraries passed quality check and were quantified before being pooled at equimolar concentration and sequenced on Illumina Nano MiSeq 2- by 150-bp paired-end mode following standard procedures. Sequenced reads that passed the quality check (Phred score ≥30) were adaptor and quality trimmed, and the remaining reads were assembled *de novo* using Megahit (v.1.2.9) with default parameter settings. Megahit generated in all cases 7 contigs with more than 1,000 bp and 100× coverage; all of these assembled contigs were compared (using BLASTn) against the entire nonredundant (nr) nucleotide and protein databases. In all cases the longest and more covered contigs were identified as MT019532.1, "Severe acute respiratory syndrome coronavirus 2 isolate BetaCoV/Wuhan/IPBCAMS-WH-04/2019, complete genome," with 99% identity and 0 gaps. The longer sequences were named hCoV-19/Italy/FVG/ICGEB_S1, _S5, _S8, and _S9 and were deposited in GISAID (see below). Sequence analysis showed an uneven coverage along the SARS-CoV-2 genome, with an average range from 126 to 7,576 reads and a mean coverage per sample of 1,169× (Fig. 1). Phylogenetic trees were inferred using the maximum likelihood method implemented in the MEGAX program using the GISAID sequences

Citation Licastro D, Rajasekharan S, Dal Monego S, Segat L, D'Agaro P, Marcello A, The Regione FVG Laboratory Group on COVID-19. 2020. Isolation and full-length genome characterization of SARS-CoV-2 from COVID-19 cases in northern Italy. J Virol 94:e00543-20. https://doi.org/10.1128/JVI.00543-20.

Editor Rozanne M. Sandri-Goldin, University of California, Irvine

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Accepted manuscript posted online 1 April 2020

Published 18 May 2020

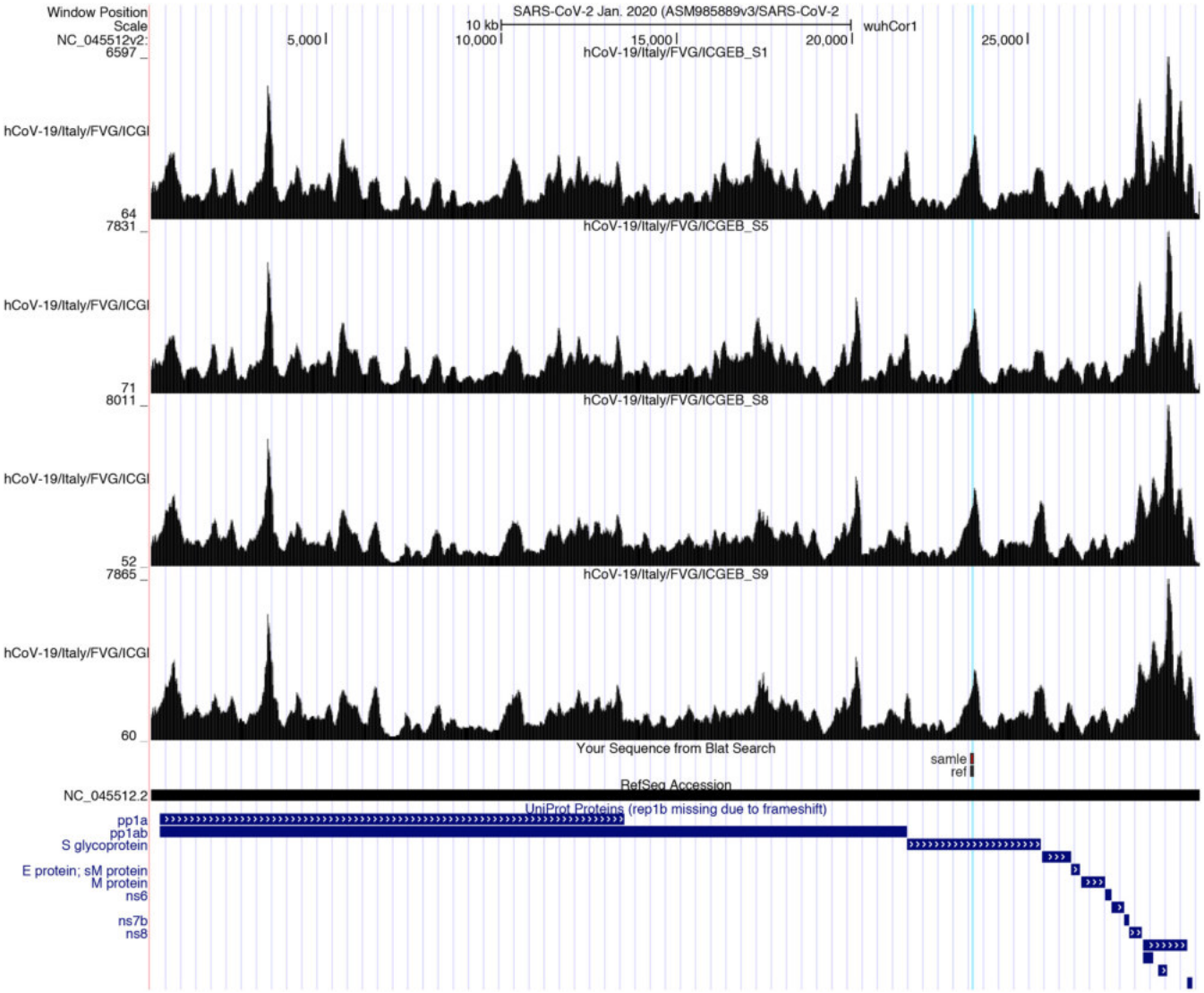

FIG 1 Sample coverage tracks from UCSC browser on ASM985889v3/SARS-CoV-2 assembly. Loaded tracks include UniProt Proteins track, RefSeq Acc track, and sample coverage tracks obtained after mapping raw reads to ASM985889v3 and converted using BEDTools genomecov function. Highlighted position refers to D614G variation in S protein revealed in all sequenced cases.

available at 03-16-2020 (6). Bootstrap support values were calculated from 500 pseudoreplicate trees of the whole data set (Fig. 2).

Despite a high burden of COVID-19 in Italy, very little information is available to date from full-length high-quality sequences. The first sequences deposited in GISAID (EPI_ISL_410545 and EPI_ISL_410546) were collected in Rome from a Chinese tourist from Hubei province who got infected before visiting Italy, and another one (EPI_ISL_412974) was from a test-positive Italian citizen returning from China. Only two sequences were reported from the Lombardy cluster (EPI_ISL_412973 and EPI_ISL_413489). In this report four additional sequences from cases epidemiologically linked to northern Italy have been examined. All infected individuals were connected to the city of Udine; S1 and S5 were from the same cluster of closely related cases, while S9 got infected probably in Lombardy and S8 visited Udine from a neighboring city (Table 1). Sequence analysis showed a good coverage along the SARS-CoV-2 genome for all four isolates (Fig. 1). Based on the marker variant S D614G, all four sequences grouped in the Bavarian rooted subclade G, which is dominant in Europe, including the sequence from Lombardy, but distinct from the three sequences mentioned above

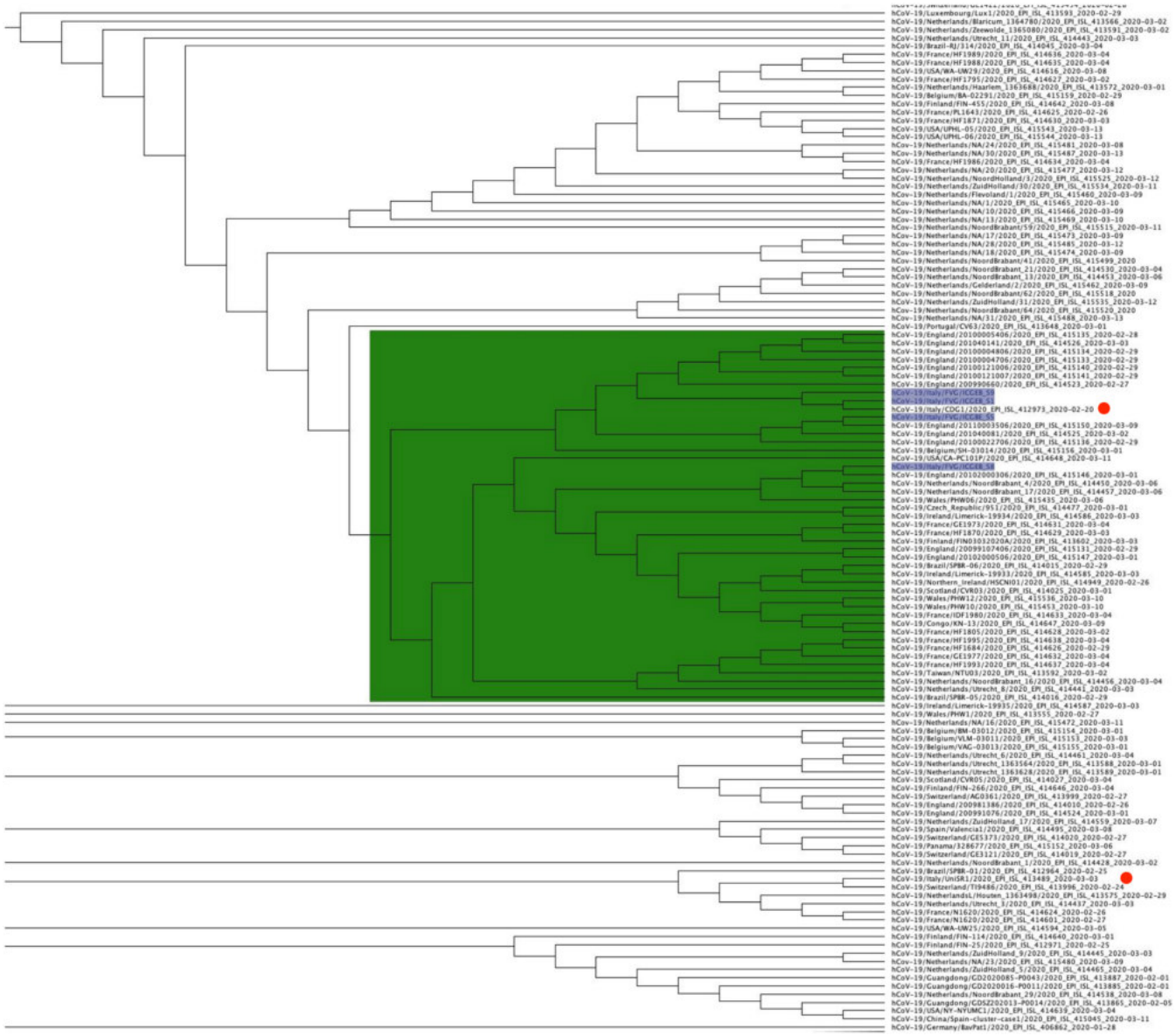

FIG 2 Maximum likelihood phylogenetic trees of nucleotide sequences from GISAID sequences available at 03-16-2020 and hCoV-19/Italy/FVG/ICGEB_S1, _S5, _S8, and _S9. A portion of the G clade based on S variation D614G is shown with indication of the phylogenetic tree branches including reporting cases (purple) and the other two deposited Lombardy sequences (red dot).

originating directly from China (7). Intriguingly, the new isolates were more closely related to EPI_ISL_412973, while EPI_ISL_413489 was more distant (Fig. 2). No evidence could be found for the putative 382-nucleotide (nt) deletion in ORF8 detected in Singapore, which has been proposed to indicate an attenuated phenotype (8).

These findings strongly urge the need for comprehensive studies that combine genomic data with epidemiological data and clinical records of symptoms from patients with COVID-19.

TABLE 1 Data for infected individuals

Sequence no.	Age (yr)	Sex	Residence	Cluster
1	59	F	Udine	Meeting in Udine
5	73	M	Udine	Meeting in Udine
8	45	M	Gorizia	Visit to Udine
9	59	M	Udine	Visit to Milan

Data availability. The longer sequences were named hCoV-19/Italy/FVG/ICGEB_S1, _S5, _S8, and _S9 and were deposited in GISAID (https://www.gisaid.org/) with accession numbers EPI_ISL_417418, EPI_ISL_417419, EPI_ISL_417421, and EPI_ISL_417423, respectively (9).

ACKNOWLEDGMENTS

Work was financed through intramural funds of the ICGEB, University of Trieste, ASUGI and the ARGO, MIUR-MISE-Region FVG Agreement Protocol signed by MIUR-MISE-Region FVG and managed by Area Science Park.

We thank Elena Samar, Fabia Petronio, and Ilaria Sandron for their excellent technical assistance.

The Regione FVG Laboratory Group on COVID-19 in Italy includes the following: Gianna Zamaro, Direzione centrale salute della Regione Friuli-Venezia Giulia, Trieste; Tolinda Gallo, Direzione centrale salute della Regione Friuli-Venezia Giulia, Trieste; Raffaella Koncan, Laboratorio di riferimento per SARS-CoV-2, Regione Friuli-Venezia Giulia, Trieste; Maurizio Ruscio, Dipartimento dei Servizi, ASUGI, Trieste; Corrado Pipan, Laboratorio di virologia, ASUFC, Udine; Francesco Curcio, Dipartimento di Laboratorio, ASUFC, Udine; and Rita de Rosa, SC Microbiologia e Virologia, ASFO, Pordenone.

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BRIEF REPORT

A Novel Coronavirus from Patients with Pneumonia in China, 2019

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SUMMARY

In December 2019, a cluster of patients with pneumonia of unknown cause was linked to a seafood wholesale market in Wuhan, China. A previously unknown betacoronavirus was discovered through the use of unbiased sequencing in samples from patients with pneumonia. Human airway epithelial cells were used to isolate a novel coronavirus, named 2019-nCoV, which formed a clade within the subgenus sarbecovirus, Orthocoronavirinae subfamily. Different from both MERS-CoV and SARS-CoV, 2019-nCoV is the seventh member of the family of coronaviruses that infect humans. Enhanced surveillance and further investigation are ongoing. (Funded by the National Key Research and Development Program of China and the National Major Project for Control and Prevention of Infectious Disease in China.)

From the NHC Key Laboratory of Biosafety, National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention (N.Z., W.W., J.S., X.Z., B.H., R.L., P.N., X.M., D.W., W.X., G.W., G.F.G., W.T.), and the Department of Infectious Diseases, Beijing Ditan Hospital, Capital Medical University (X.L.) — both in Beijing; Wuhan Jinyintan Hospital (D.Z.), the Division for Viral Disease Detection, Hubei Provincial Center for Disease Control and Prevention (B.Y., F.Z.), and the Center for Biosafety Mega-Science, Chinese Academy of Sciences (W.T.) — all in Wuhan; and the Shandong First Medical University and Shandong Academy of Medical Sciences, Jinan, China (W.S.). Address reprint requests to Dr. Tan at the NHC Key Laboratory of Biosafety, National Institute for Viral Disease Control and Prevention, China CDC, 155 Changbai Road, Changping District, Beijing 102206, China; or at tanwj@ivdc.chinacdc.cn, Dr. Gao at the National Institute for Viral Disease Control and Prevention, China CDC, Beijing 102206, China, or at gaof@im.ac.cn, or Dr. Wu at the NHC Key Laboratory of Biosafety, National Institute for Viral Disease Control and Prevention, China CDC, Beijing 102206, China, or at wugz@ivdc.chinacdc.cn.

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This article was published on January 24, 2020, and updated on January 29, 2020, at NEJM.org.

N Engl J Med 2020;382:727-33.
DOI: 10.1056/NEJMoa2001017

Emerging and reemerging pathogens are global challenges for public health.[1] Coronaviruses are enveloped RNA viruses that are distributed broadly among humans, other mammals, and birds and that cause respiratory, enteric, hepatic, and neurologic diseases.[2,3] Six coronavirus species are known to cause human disease.[4] Four viruses — 229E, OC43, NL63, and HKU1 — are prevalent and typically cause common cold symptoms in immunocompetent individuals.[4] The two other strains — severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) — are zoonotic in origin and have been linked to sometimes fatal illness.[5] SARS-CoV was the causal agent of the severe acute respiratory syndrome outbreaks in 2002 and 2003 in Guangdong Province, China.[6-8] MERS-CoV was the pathogen responsible for severe respiratory disease outbreaks in 2012 in the Middle East.[9] Given the high prevalence and wide distribution of coronaviruses, the large genetic diversity and frequent recombination of their genomes, and increasing human–animal interface activities, novel coronaviruses are likely to emerge periodically in humans owing to frequent cross-species infections and occasional spillover events.[5,10]

In late December 2019, several local health facilities reported clusters of patients with pneumonia of unknown cause that were epidemiologically linked to a seafood and wet animal wholesale market in Wuhan, Hubei Province, China.[11] On December 31, 2019, the Chinese Center for Disease Control and Prevention (China CDC) dispatched a rapid response team to accompany Hubei provincial and Wuhan city health authorities and to conduct an epidemiologic and etiologic investigation. We report the results of this investigation, identifying the source of the pneumonia

clusters, and describe a novel coronavirus detected in patients with pneumonia whose specimens were tested by the China CDC at an early stage of the outbreak. We also describe clinical features of the pneumonia in two of these patients.

METHODS

VIRAL DIAGNOSTIC METHODS

Four lower respiratory tract samples, including bronchoalveolar-lavage fluid, were collected from patients with pneumonia of unknown cause who were identified in Wuhan on December 21, 2019, or later and who had been present at the Huanan Seafood Market close to the time of their clinical presentation. Seven bronchoalveolar-lavage fluid specimens were collected from patients in Beijing hospitals with pneumonia of known cause to serve as control samples. Extraction of nucleic acids from clinical samples (including uninfected cultures that served as negative controls) was performed with a High Pure Viral Nucleic Acid Kit, as described by the manufacturer (Roche). Extracted nucleic acid samples were tested for viruses and bacteria by polymerase chain reaction (PCR), using the RespiFinderSmart22kit (PathoFinder BV) and the LightCycler 480 real-time PCR system, in accordance with manufacturer instructions.[12] Samples were analyzed for 22 pathogens (18 viruses and 4 bacteria) as detailed in the Supplementary Appendix. In addition, unbiased, high-throughput sequencing, described previously,[13] was used to discover microbial sequences not identifiable by the means described above. A real-time reverse transcription PCR (RT-PCR) assay was used to detect viral RNA by targeting a consensus RdRp region of pan β-CoV, as described in the Supplementary Appendix.

ISOLATION OF VIRUS

Bronchoalveolar-lavage fluid samples were collected in sterile cups to which virus transport medium was added. Samples were then centrifuged to remove cellular debris. The supernatant was inoculated on human airway epithelial cells,[13] which had been obtained from airway specimens resected from patients undergoing surgery for lung cancer and were confirmed to be special-pathogen-free by NGS.[14]

Human airway epithelial cells were expanded on plastic substrate to generate passage-1 cells and were subsequently plated at a density of 2.5×10^5 cells per well on permeable Transwell-COL (12-mm diameter) supports. Human airway epithelial cell cultures were generated in an air–liquid interface for 4 to 6 weeks to form well-differentiated, polarized cultures resembling in vivo pseudostratified mucociliary epithelium.[13]

Prior to infection, apical surfaces of the human airway epithelial cells were washed three times with phosphate-buffered saline; 150 μl of supernatant from bronchoalveolar-lavage fluid samples was inoculated onto the apical surface of the cell cultures. After a 2-hour incubation at 37°C, unbound virus was removed by washing with 500 μl of phosphate-buffered saline for 10 minutes; human airway epithelial cells were maintained in an air–liquid interface incubated at 37°C with 5% carbon dioxide. Every 48 hours, 150 μl of phosphate-buffered saline was applied to the apical surfaces of the human airway epithelial cells, and after 10 minutes of incubation at 37°C the samples were harvested. Pseudostratified mucociliary epithelium cells were maintained in this environment; apical samples were passaged in a 1:3 diluted vial stock to new cells. The cells were monitored daily with light microscopy, for cytopathic effects, and with RT-PCR, for the presence of viral nucleic acid in the supernatant. After three passages, apical samples and human airway epithelial cells were prepared for transmission electron microscopy.

TRANSMISSION ELECTRON MICROSCOPY

Supernatant from human airway epithelial cell cultures that showed cytopathic effects was collected, inactivated with 2% paraformaldehyde for at least 2 hours, and ultracentrifuged to sediment virus particles. The enriched supernatant was negatively stained on film-coated grids for examination. Human airway epithelial cells showing cytopathic effects were collected and fixed with 2% paraformaldehyde–2.5% glutaraldehyde and were then fixed with 1% osmium tetroxide dehydrated with grade ethanol embedded with PON812 resin. Sections (80 nm) were cut from resin block and stained with uranyl acetate and lead citrate, separately. The negative stained grids and ultrathin sections were observed under transmission electron microscopy.

VIRAL GENOME SEQUENCING

RNA extracted from bronchoalveolar-lavage fluid and culture supernatants was used as a template to clone and sequence the genome. We used a combination of Illumina sequencing and nanopore sequencing to characterize the virus genome. Sequence reads were assembled into contig maps (a set of overlapping DNA segments) with the use of CLC Genomics software, version 4.6.1 (CLC Bio). Specific primers were subsequently designed for PCR, and 5′- or 3′-RACE (rapid amplification of cDNA ends) was used to fill genome gaps from conventional Sanger sequencing. These PCR products were purified from gels and sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit and a 3130XL Genetic Analyzer, in accordance with the manufacturers' instructions.

Multiple-sequence alignment of the 2019-nCoV and reference sequences was performed with the use of Muscle. Phylogenetic analysis of the complete genomes was performed with RAxML (13) with 1000 bootstrap replicates and a general time-reversible model used as the nucleotide substitution model.

RESULTS

PATIENTS

Three adult patients presented with severe pneumonia and were admitted to a hospital in Wuhan on December 27, 2019. Patient 1 was a 49-year-old woman, Patient 2 was a 61-year-old man, and Patient 3 was a 32-year-old man. Clinical profiles were available for Patients 1 and 2. Patient 1 reported having no underlying chronic medical conditions but reported fever (temperature, 37°C to 38°C) and cough with chest discomfort on December 23, 2019. Four days after the onset of illness, her cough and chest discomfort worsened, but the fever was reduced; a diagnosis of pneumonia was based on computed tomographic (CT) scan. Her occupation was retailer in the seafood wholesale market. Patient 2 initially reported fever and cough on December 20, 2019; respiratory distress developed 7 days after the onset of illness and worsened over the next 2 days (see chest radiographs, Fig. 1), at which time mechanical ventilation was started. He had been a frequent visitor to the seafood wholesale market. Patients 1 and 3 recovered and were discharged from the hospital on January 16, 2020. Patient 2 died on January 9, 2020. No biopsy specimens were obtained.

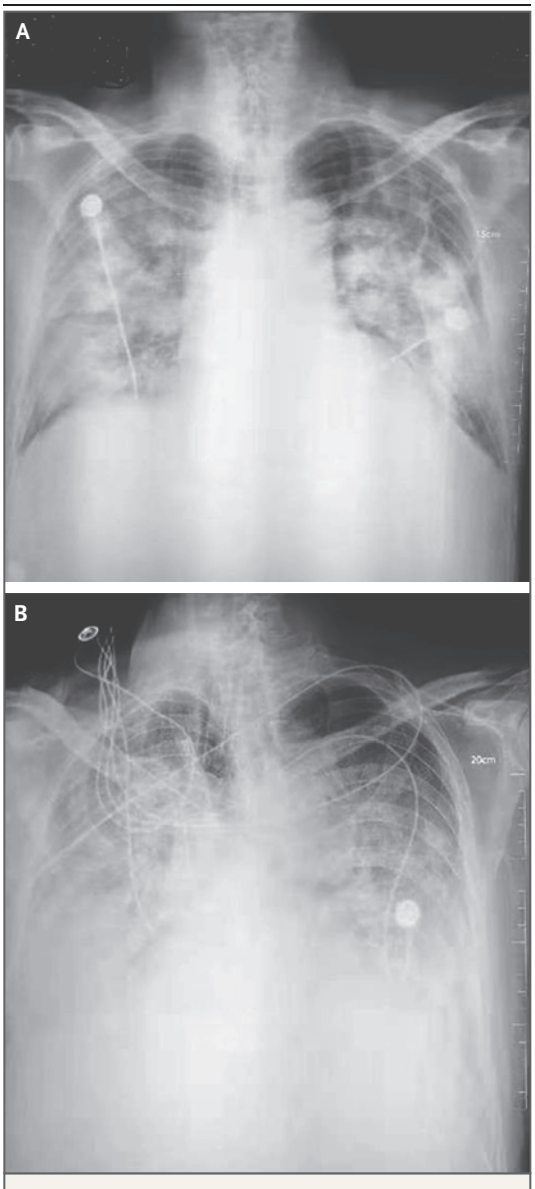

Figure 1. Chest Radiographs.

Shown are chest radiographs from Patient 2 on days 8 and 11 after the onset of illness. The trachea was intubated and mechanical ventilation instituted in the period between the acquisition of the two images. Bilateral fluffy opacities are present in both images but are increased in density, profusion, and confluence in the second image; these changes are most marked in the lower lung fields. Changes consistent with the accumulation of pleural liquid are also visible in the second image.

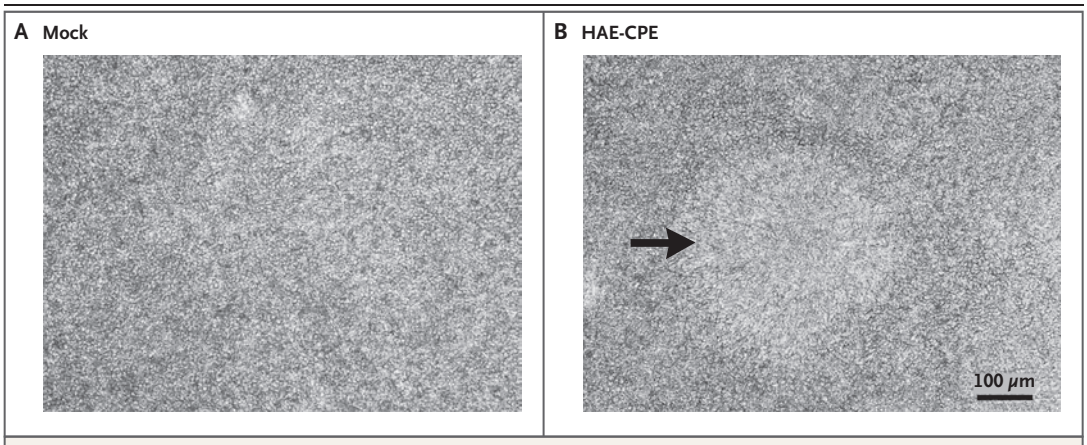

Figure 2. Cytopathic Effects in Human Airway Epithelial Cell Cultures after Inoculation with 2019-nCoV.

DETECTION AND ISOLATION OF A NOVEL CORONAVIRUS

Three bronchoalveolar-lavage samples were collected from Wuhan Jinyintan Hospital on December 30, 2019. No specific pathogens (including HCoV-229E, HCoV-NL63, HCoV-OC43, and HCoV-HKU1) were detected in clinical specimens from these patients by the RespiFinderSmart-22kit. RNA extracted from bronchoalveolar-lavage fluid from the patients was used as a template to clone and sequence a genome using a combination of Illumina sequencing and nanopore sequencing. More than 20,000 viral reads from individual specimens were obtained, and most contigs matched to the genome from lineage B of the genus betacoronavirus — showing more than 85% identity with a bat SARS-like CoV (bat-SL-CoVZC45, MG772933.1) genome published previously. Positive results were also obtained with use of a real-time RT-PCR assay for RNA targeting to a consensus RdRp region of pan β-CoV (although the cycle threshold value was higher than 34 for detected samples). Virus isolation from the clinical specimens was performed with human airway epithelial cells and Vero E6 and Huh-7 cell lines. The isolated virus was named 2019-nCoV.

To determine whether virus particles could be visualized in 2019-nCoV–infected human airway epithelial cells, mock-infected and 2019-nCoV–infected human airway epithelial cultures were examined with light microscopy daily and with transmission electron microscopy 6 days after inoculation. Cytopathic effects were observed 96 hours after inoculation on surface layers of human airway epithelial cells; a lack of cilium beating was seen with light microcopy in the center of the focus (Fig. 2). No specific cytopathic effects were observed in the Vero E6 and Huh-7 cell lines until 6 days after inoculation.

Electron micrographs of negative-stained 2019-nCoV particles were generally spherical with some pleomorphism (Fig. 3). Diameter varied from about 60 to 140 nm. Virus particles had quite distinctive spikes, about 9 to 12 nm, and gave virions the appearance of a solar corona. Extracellular free virus particles and inclusion bodies filled with virus particles in membrane-bound vesicles in cytoplasm were found in the human airway epithelial ultrathin sections. This observed morphology is consistent with the Coronaviridae family.

To further characterize the virus, de novo sequences of 2019-nCoV genome from clinical specimens (bronchoalveolar-lavage fluid) and human airway epithelial cell virus isolates were obtained by Illumina and nanopore sequencing. The novel coronavirus was identified from all three patients. Two nearly full-length coronavirus sequences were obtained from bronchoalveolar-lavage fluid (BetaCoV/Wuhan/IVDC-HB-04/2020, BetaCoV/Wuhan/IVDC-HB-05/2020|EPI_ISL_402121), and one full-length sequence was obtained from a virus isolated from a patient (BetaCoV/Wuhan/IVDC-HB-01/2020|EPI_ISL_402119). Complete genome sequences of the three novel coronaviruses were submitted to GISAID (BetaCoV/Wuhan/IVDC-HB-01/2019, accession ID: EPI_ISL_402119; BetaCoV/Wuhan/IVDC-HB-04/2020, accession ID: EPI_ISL_402120; BetaCoV/Wuhan/IVDC-HB-05/2019,

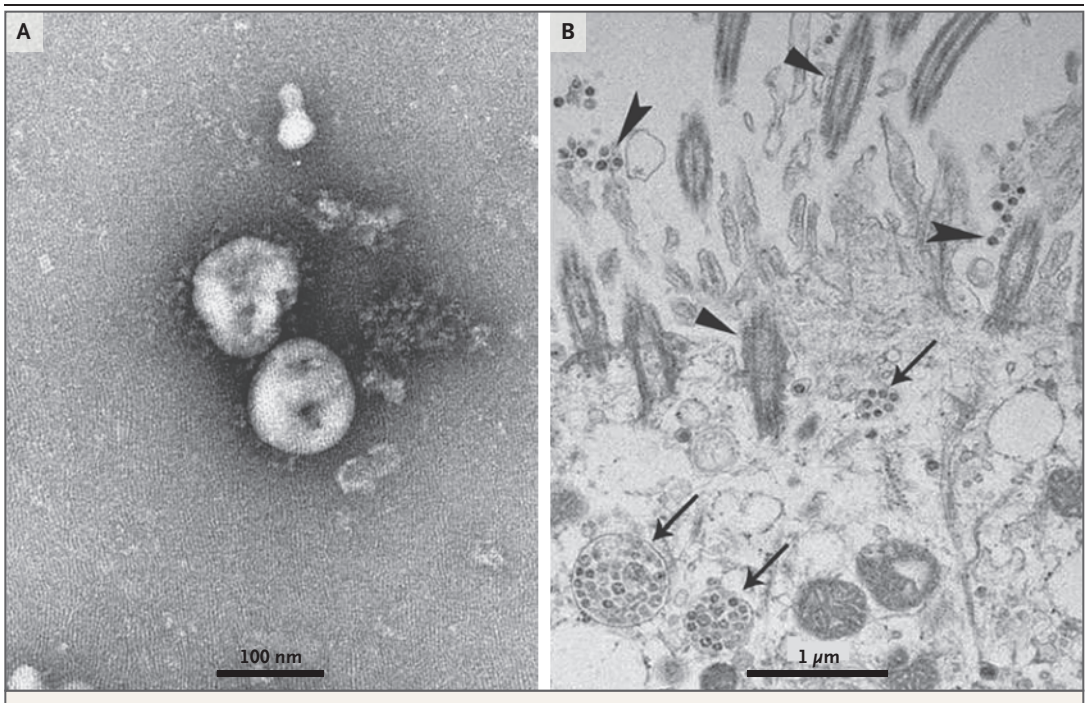

Figure 3. Visualization of 2019-nCoV with Transmission Electron Microscopy.
Negative-stained 2019-nCoV particles are shown in Panel A, and 2019-nCoV particles in the human airway epithelial cell ultrathin sections are shown in Panel B. Arrowheads indicate extracellular virus particles, arrows indicate inclusion bodies formed by virus components, and triangles indicate cilia.

accession ID: EPI_ISL_402121) and have a 86.9% nucleotide sequence identity to a previously published bat SARS-like CoV (bat-SL-CoVZC45, MG772933.1) genome. The three 2019-nCoV genomes clustered together within the sarbecovirus subgenus, which shows the typical betacoronavirus organization: a 5′ untranslated region (UTR), replicase complex (orf1ab), S gene, E gene, M gene, N gene, 3′ UTR, and several unidentified nonstructural open reading frames.

Although 2019-nCoV is similar to some betacoronaviruses detected in bats (Fig. 4), it is distinct from SARS-CoV and MERS-CoV. The three 2019-nCoV coronaviruses from Wuhan, together with two bat-derived SARS-like strains, ZC45 and ZXC21, form a distinct clade. SARS-CoV strains from humans and genetically similar SARS-like coronaviruses from bats collected from southwestern China formed another clade within the subgenus sarbecovirus. Since the sequence identity in conserved replicase domains (ORF 1ab) is less than 90% between 2019-nCoV and other members of betacoronavirus, the 2019-nCoV — the likely causative agent of the viral pneumonia in Wuhan — is a novel betacoronavirus belonging to the sarbecovirus subgenus of Coronaviridae family.

DISCUSSION

We report a novel CoV (2019-nCoV) that was identified in hospitalized patients in Wuhan, China, in December 2019 and January 2020. Evidence for the presence of this virus includes identification in bronchoalveolar-lavage fluid in three patients by whole-genome sequencing, direct PCR, and culture. The illness likely to have been caused by this CoV was named "novel coronavirus-infected pneumonia" (NCIP). Complete genomes were submitted to GISAID. Phylogenetic analysis revealed that 2019-nCoV falls into the genus betacoronavirus, which includes coronaviruses (SARS-CoV, bat SARS-like CoV, and others) discovered in humans, bats, and other wild animals.[15] We report isolation of the virus and the initial description of its specific cytopathic effects and morphology.

Molecular techniques have been used successfully to identify infectious agents for many years. Unbiased, high-throughput sequencing is

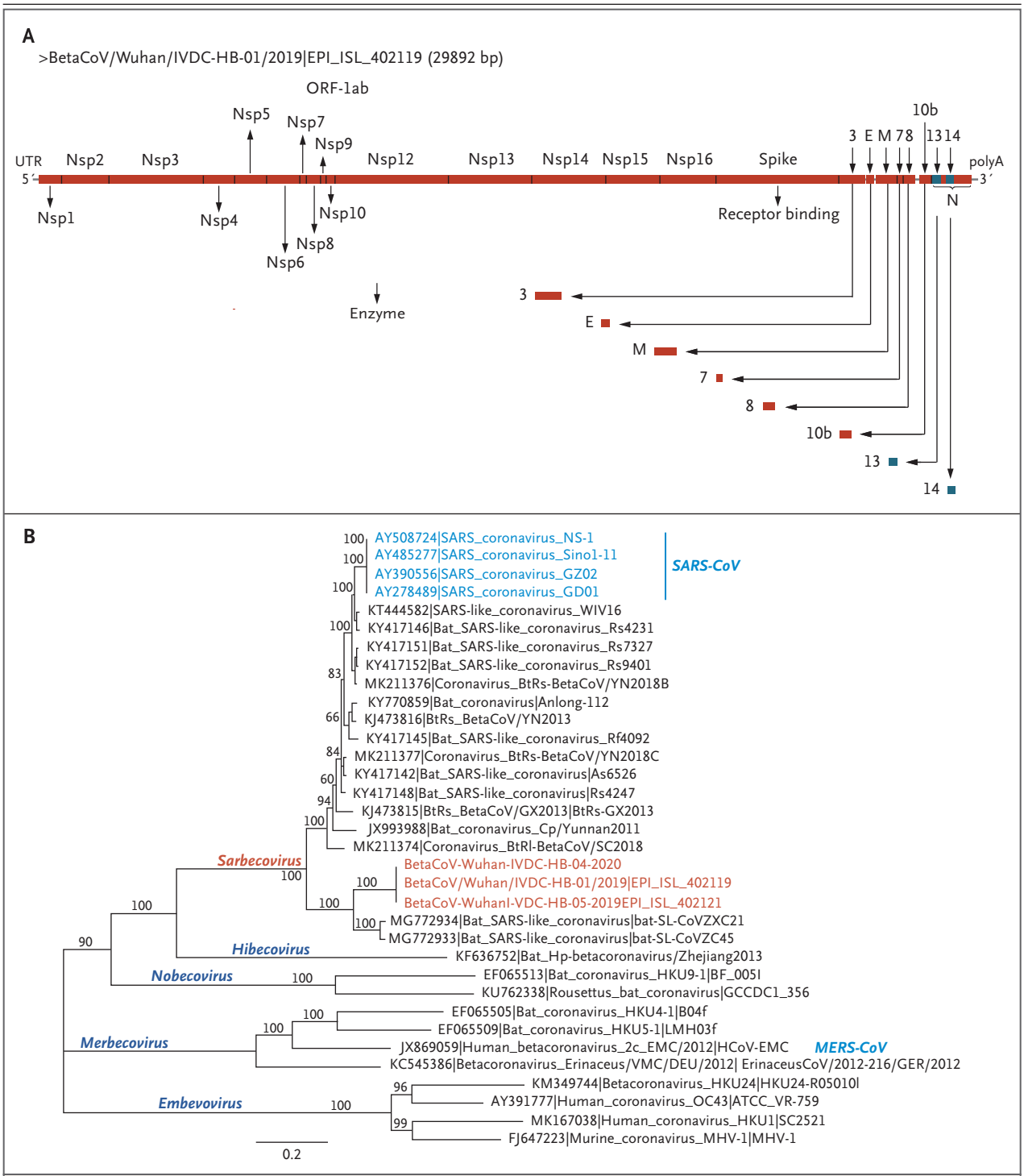

Figure 4. Schematic of 2019-nCoV and Phylogenetic Analysis of 2019-nCoV and Other Betacoronavirus Genomes.

Shown are a schematic of 2019-nCoV (Panel A) and full-length phylogenetic analysis of 2019-nCoV and other betacoronavirus genomes in the Orthocoronavirinae subfamily (Panel B).

a powerful tool for the discovery of pathogens.[14,16] Next-generation sequencing and bioinformatics are changing the way we can respond to infectious disease outbreaks, improving our understanding of disease occurrence and transmission, accelerating the identification of pathogens, and promoting data sharing. We describe in this report the use of molecular techniques and unbiased DNA sequencing to discover a novel betacoronavirus that is likely to have been the cause of severe pneumonia in three patients in Wuhan, China.

Although establishing human airway epithelial cell cultures is labor intensive, they appear to be a valuable research tool for analysis of human respiratory pathogens.[13] Our study showed that initial propagation of human respiratory secretions onto human airway epithelial cell cultures, followed by transmission electron microscopy and whole genome sequencing of culture supernatant, was successfully used for visualization and detection of new human coronavirus that can possibly elude identification by traditional approaches.

Further development of accurate and rapid methods to identify unknown respiratory pathogens is still needed. On the basis of analysis of three complete genomes obtained in this study, we designed several specific and sensitive assays targeting ORF1ab, N, and E regions of the 2019-nCoV genome to detect viral RNA in clinical specimens. The primer sets and standard operating procedures have been shared with the World Health Organization and are intended for surveillance and detection of 2019-nCoV infection globally and in China. More recent data show 2019-nCoV detection in 830 persons in China.[17]

Although our study does not fulfill Koch's postulates, our analyses provide evidence implicating 2019-nCoV in the Wuhan outbreak. Additional evidence to confirm the etiologic significance of 2019-nCoV in the Wuhan outbreak include identification of a 2019-nCoV antigen in the lung tissue of patients by immunohistochemical analysis, detection of IgM and IgG antiviral antibodies in the serum samples from a patient at two time points to demonstrate seroconversion, and animal (monkey) experiments to provide evidence of pathogenicity. Of critical importance are epidemiologic investigations to characterize transmission modes, reproduction interval, and clinical spectrum resulting from infection to inform and refine strategies that can prevent, control, and stop the spread of 2019-nCoV.

This work was supported by grants from the National Key Research and Development Program of China (2016YFD0500301) and the National Major Project for Control and Prevention of Infectious Disease in China (2018ZX10101002).

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

We thank Dr. Zhongjie Li, Dr. Guangxue He, Dr. Lance Rodewald, Yu Li, Fei Ye, Li Zhao, Weimin Zhou, Jun Liu, Yao Meng, Huijuan Wang, and many staff members at the China CDC for their contributions and assistance in this preparation and submission of an earlier version of the manuscript.

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J Korean Med Sci. 2020 Feb 24;35(7):e84
https://doi.org/10.3346/jkms.2020.35.e84
eISSN 1598-6357·pISSN 1011-8934

JKMS

Brief Communication

Infectious Diseases, Microbiology & Parasitology

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Virus Isolation from the First Patient with SARS-CoV-2 in Korea

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OPEN ACCESS

Received: Feb 11, 2020
Accepted: Feb 17, 2020

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ABSTRACT

Novel coronavirus (SARS-CoV-2) is found to cause a large outbreak started from Wuhan since December 2019 in China and SARS-CoV-2 infections have been reported with epidemiological linkage to China in 25 countries until now. We isolated SARS-CoV-2 from the oropharyngeal sample obtained from the patient with the first laboratory-confirmed SARS-CoV-2 infection in Korea. Cytopathic effects of SARS-CoV-2 in the Vero cell cultures were confluent 3 days after the first blind passage of the sample. Coronavirus was confirmed with spherical particle having a fringe reminiscent of crown on transmission electron microscopy. Phylogenetic analyses of whole genome sequences showed that it clustered with other SARS-CoV-2 reported from Wuhan.

Keywords: 2019 Novel Coronavirus; Culture; Microscopy, Electron; Phylogeny; Korea

Coronaviruses (CoVs), within the order Nidovirales, are enveloped, single-strand, positive-sense RNA viruses with a large genome of approximately 30 kbp in length. CoV was cultured for the first time in human embryonic tracheal organ cultures by Tyrrell and Bynoe in 1965,[1] and it was named as 'corona' due to crown-like appearance of the surface projections on electron microscopy. All CoVs develop only in the cytoplasm of infected cells, bud into cytoplasmic vesicles, and then extrude in virus particles of 70–80 nm in diameter by exocytic secretory pathway.

Among four genera of CoVs, beta-CoV includes five subgenus—embevovirus, sarbecovirus including severe acute respiratory syndrome (SARS)-CoV, merbecovirus including Middle East respiratory syndrome (MERS)-CoV, nobecovirus, and hibecovirus. Because CoVs can infect a variety of animals, SARS-CoV and MERS-CoV crossed the species barriers.

Since December 2019, 2019 novel CoV (SARS-CoV-2) has been making a large outbreak involving 49,053 laboratory-confirmed patients and 1,383 mortality in 25 countries including

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Disclosure

The authors have no potential conflicts of interest to disclose.

Author Contributions

Conceptualization: Oh MD, Park WB. Data curation: Park WB, Kwon NJ, Choi SJ, Yun J, Lee GW, Kim JY. Formal analysis: Oh MD, Park WB, Seong MW, Kim NJ, Seo JS. Writing - original draft: Park WB, Kwon NJ, Kang CK. Writing - review & editing: Seong MW, Choe PG, Kim NJ, Oh MD.

Korea until February 14, 2020.[2] In the outbreak situation, isolation of causative virus is indispensable for developing and evaluating diagnostic tools, therapeutics, and vaccine candidates. SARS-CoV-2 was first isolated using human airway epithelial cells and it was classified into the subgenus sarbecovirus of beta-CoVs by phylogenetic analyses of the gene sequences.[3] Both the SARS-CoV and the MERS-CoV were initially isolated and grew readily in Vero cells.[4,5] Here, we report the isolation of SARS-CoV-2 using Vero cells from a patient entering Korea from Wuhan, China.

The patient with the first laboratory-confirmed SARS-CoV-2 infection in Korea is published previously.[6] Briefly, a 35-year-old woman developed fever, chill, and myalgia on January 18, 2020, and arrived at the Incheon airport from Wuhan on the next day. After laboratory-confirmed diagnosis of SARS-CoV-2 infection, she developed nasal congestion, cough, and sputum. Oxygen supplementation was started on day 4 of her illness, and her oxygen requirement increased to 6 L/min on day 7 of illness. Fever persisted for ten days and her maximum body temperature during her illness was 38.9°C on day 7 of illness.

The patient's oropharyngeal samples were obtained by using UTM™ kit containing 1 mL of viral transport media (Copan Diagnostics Inc., Murrieta, CA, USA) on day 7 of her illness. We inoculated monolayers of Vero cells (*ATCC*® CCL-81™) with the samples and cultured the cells at 37°C in a 5% carbon dioxide atmosphere. Until 5 days after inoculation, cytopathic effects were not distinct, which is compatible with the previous findings that no specific cytopathic effects were observed in the Vero E6 cells until 6 days after inoculation in the report about first isolation of SARS-CoV-2.[3] Five days after inoculation, we did blind passage of culture supernatant into T-25 culture flask (ThermoFisher Scientific Inc., Waltham, MA, USA) with monolayers of Vero cells, and cytopathic effects consisting of rounding and detachment of cells were observed in the whole area of the T-25 flask 3 days after the first blind passage (**Fig. 1A and B**).

In order to observe virus particles, Vero cell monolayer showing the cytopathic effects was fixed as previously described.[7] It was cut on ultramicrotome (RMC MT-XL; RMC Boeckeler, Tucson, AZ, USA) at 65 nm. Ultrathin sections were stained with saturated 4% uranyl acetate and 1% lead citrate before examination with a transmission electron microscope (JEM-1400; JEOL USA Inc., Peabody, MA, USA) at 80 kV. Spherical particles with crown-like spikes ranging 66 to 81 nm in diameter were observed within the cytoplasmic vesicles and in the extracellular space adjacent to cell membrane (**Fig. 1C and D**).

For whole genome sequencing of the virus isolate (BetaCoV/Korea/SNU01/2020), culture supernatant of Vero cells infected was used for RNA extraction. RNA was extracted by using QIAamp viral RNA mini kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's instructions. RNA libraries were prepared using TruSeq Stranded Total RNA Kit (catalog No. 20020596; Illumina, San Diego, CA, USA) according to the manufacturer protocol. Sequencing was performed on an Illumina Nextseq 500 platform, produced on average a total of 150 million reads, 150 bp per sample, as per the manufacturer's instructions in Macrogen Inc. (Seoul, Korea).[8-10] FASTQ was used to trim the adapter and remove low quality bases and reads. Qualified reads were mapped to NC_045512, a SARS-CoV-2 genome reference using Burrows-Wheeler Aligner (v0.7.12-r1039), and a bam file was produced.[11] In this bam file, the variation was confirmed by comparing with genome using SAMtools (v1.3.1).[12] For genome-base phylogeny analysis, 37 strains including BetaCoV/Korea/KCDC03/2020 were used in combination with BetaCoV/Korea/SNU01/2020. The sequences used for analysis were

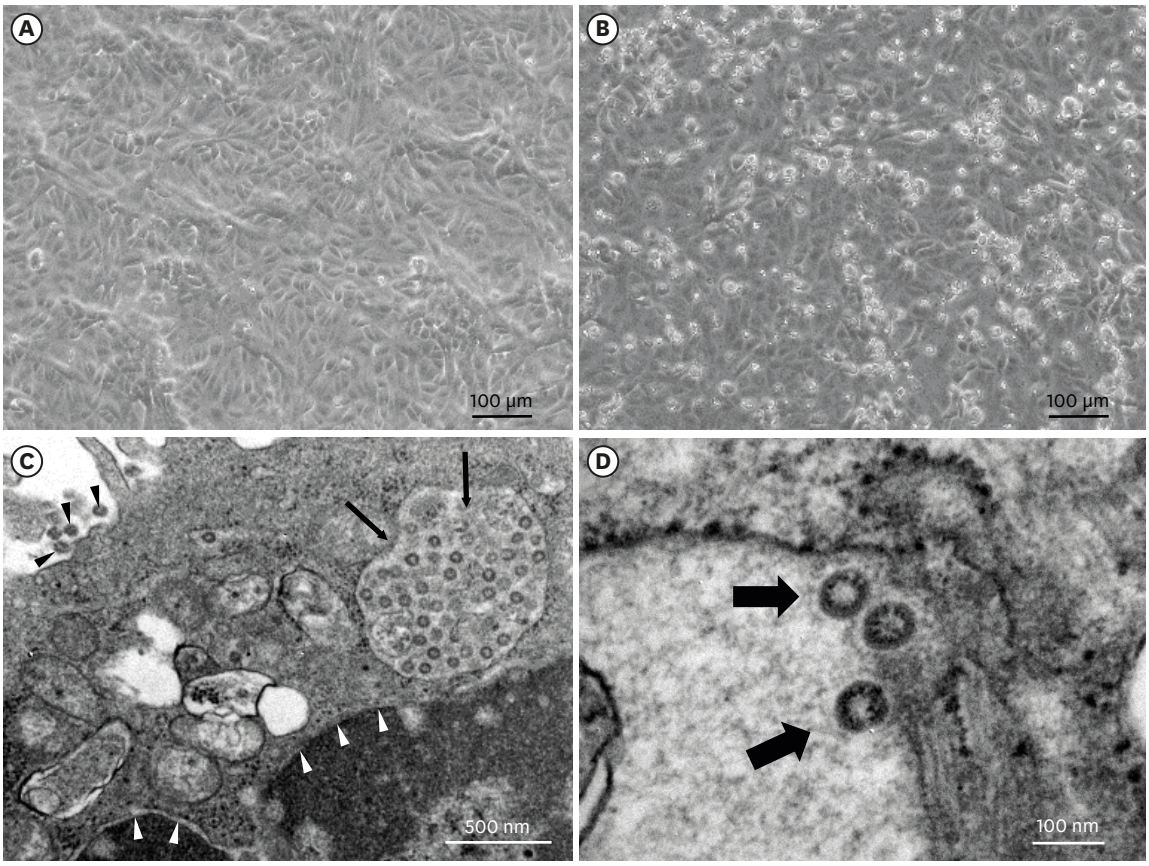

Fig. 1. Cytopathic effects of SARS-CoV-2 in Vero cell cultures and electron microscopy image of SARS-CoV-2. Vero cells were inoculated with oropharyngeal swab sample. (**A**) Vero cell cultures in negative control. (**B**) Cytopathic effects consisting of rounding and detachment of cells in Vero cell cultures 3 days after the first blind passage. (**C**, **D**) Transmission electron microscopy image of Vero cells infected with SARS-CoV-2. White arrow head denotes nuclear membrane, black arrow head extracellular virus particles, and thin black arrow cytoplasmic vesicle including virus components (**C**). Thick black arrow denotes magnified virus particles with crown-like spikes (**D**).
SARS = severe acute respiratory syndrome, CoV = coronavirus.

downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) and GISAID (http://www.gisaid.org). The 37 strain genomes were multiple-sequence aligned using MAFFT (v7.450), a sequence alignment tool, and were used to generate phylogenetic tree.[13] Phylogenetic analysis of the aligned sequence was performed with 1,000 bootstrap replicates using MEGAX and a general time-reversible model used as the nucleotide substitution model.[14]

Next-generation sequencing of BetaCoV/Korea/SNU01/2020 (GenBank accession no. MT039890) revealed 9 mutations compared to the NC_045512 reference genome isolated from Wuhan (**Table 1**). Most of the mutations in our isolate consisted of 70% alternative genes and 30% reference genes (NC_045512). Five variants were found in ORF1ab, one variant in S gene, two variants in ORF3a, and one variant in E gene. Of the nine mutations, six also showed changes in amino acids. When comparing our isolate with the one isolated from the Korea Centers for Disease Control and Prevention (BetaCoV/Korea/KCDC03/2020), 12 variants including the above 9 mutations were found. These mutations may occur by cell culture-adaptation in that our culture isolate was obtained after first blind passage, or by micro-evolution of SARS-CoV-2 before acquisition in Wuhan. Because those genome sequences are quite homologous each other, it is difficult to validate these two hypothesis.

https://doi.org/10.3346/jkms.2020.35.e84

Table 1. Genetic variations of our isolate (BetaCoV/Korea/SNU01/2020) compared to the NC_045512 reference genome of a SARS-CoV-2 isolated in Wuhan

Position	Gene	Ref	Alt	Ref AA	Alt AA	Total depth	Ref, %	Alt, %
2971	ORF1ab	G	T	Met	Ile	7917	31	69
6031	ORF1ab	C	T	Asn	Asn	7966	5	95
12115	ORF1ab	C	T	Ser	Ser	7875	33	67
15597	ORF1ab	T	C	Tyr	Tyr	7977	4	96
20936	ORF1ab	C	T	Thr	Met	7984	31	69
22224	S	C	G	Ser	Trp	7993	33	67
25775	ORF3a	G	T	Trp	Leu	7995	32	68
26144	ORF3a	G	T	Gly	Val	7885	0	100
26354	E	T	A	Leu	His	7609	36	64

SARS = severe acute respiratory syndrome, CoV = coronavirus, Ref = reference, Alt = alternative, AA = amino acid.

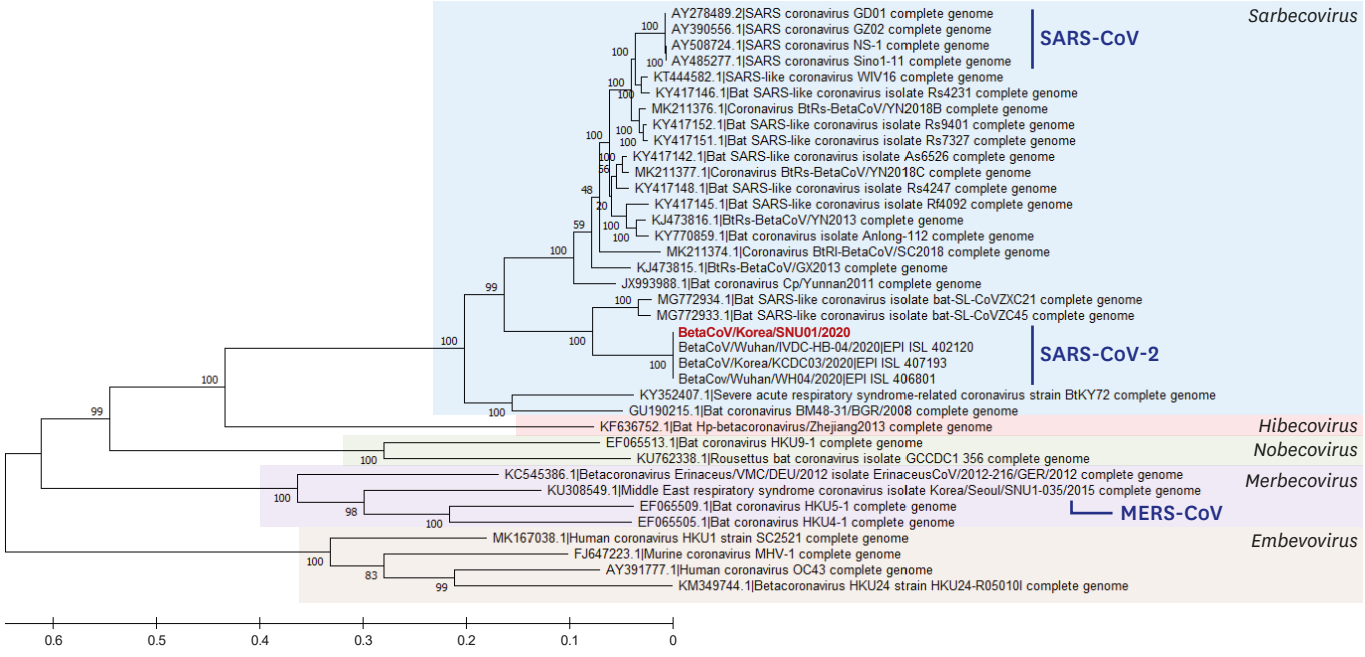

Fig. 2. Phylogenetic analysis of full-length genomes of SARS-CoV-2 including our isolate and other representative beta-CoVs.
SARS = severe acute respiratory syndrome, CoV = coronavirus, MERS = Middle East respiratory syndrome.

The phylogenetic tree was analyzed using 37 genome information including CoVs isolated from Korea and Wuhan (**Fig. 2**). As a result, it was confirmed that our isolate is a beta-CoV belonging to the subgenus sarbecovirus, and that it closely clustered with other SARS-CoV-2 isolated from Korea and Wuhan.

In summary, we isolated SARS-CoV-2 using Vero cells from the first laboratory-confirmed SARS-CoV-2-infected patient in Korea. Phylogenetic analyses of the whole genome sequences showed that it clustered with other SARS-CoV-2 reported from Wuhan, China.

ACKNOWLEDGMENTS

We thank Eun Kyung Choi (Department of Pathology, Seoul National University Hospital) for her technical assistance in electron microscopy.

https://doi.org/10.3346/jkms.2020.35.e84

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

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

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

Keywords: Coronavirus, 2019 -nCoV, SARS-CoV-2, COVID-19

ABSTRACT

The etiologic agent of the outbreak of pneumonia in Wuhan China was identified as severe acute respiratory syndrome associated coronavirus 2 (SARS-CoV-2) in January, 2020. 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 replicates to high titer 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 of coronaviruses mediates virus binding and cell entry. Initial characterization of SARS-CoV-2 spike indicate that it binds the same receptor as SARS-CoV, ACE2, which is expressed in both upper and lower human respiratory tracts (8). The unprecedented rapidity of spread of this outbreak represents a critical need for reference reagents. 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.

RESULTS and DISCUSSION

A patient was identified with confirmed COVID-19 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 inoculation 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 P1 viral lysates were used for nucleic acid extraction to confirm the presence of SARS-CoV-2 using the CDC molecular diagnostic assay (1). The C_ts of three different nucleic acid extractions 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. The extracts were also tested for the presence of 33 additional different respiratory pathogens with the fast track 33 assay. No other pathogens were detected. Identity was additionally supported by thin section electron microscopy (Figure 1D). We observed a 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 clinical specimen 100% (Genbank accession MN985325).

After the second passage, OP and NP specimens were not cultured separately. 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 sequence of SARS-CoV-2 has an RRAR insertion at the S1-S2 interface that may be cleaved by furin (10). 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 (11). 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 (12, 13). 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 lines and types susceptible to infection 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 and suggest other common culture systems including MDCK, HeLa, HEP-2, MRC-5 cells, and embryonated eggs are unlikely to support SARS-CoV-2 replication (14-16). 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 polyclonal sera 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 due to predicted insertion of a furin cleavage site in SARS-CoV-2 (10). 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.

Finally, we evaluated the replication kinetics of SARS-CoV-2 in a multi-step growth curve. Briefly, Vero CCL-81 and HUH7.0 cells were infected with SARS-CoV-2 at a low multiplicity of infection (MOI 0.1) and viral replication evaluated every 6 hours for 72 hours post inoculation, with separate harvests in the cell-associated and supernatant compartments (**Fig. 4**). Similar to SARS-CoV, SARS-CoV-2 replicated rapidly in Vero cells after an initial eclipse phase, achieving 10^5 TCID50 / ml by 24 hours post infection and peaking at > 10^6 TCID50 / ml. Similar titers were observed in cell-associated and supernatant compartments indicating efficient egress. Despite peak viral titers by 48 hours post-inoculation, significant CPE was not observed until 60 hours post inoculation and peaked at 72 hours post-inoculation, indicating scientists should harvest infected monolayers before peak CPE is observed. Replication in HUH7.0 cells also increased quickly after an initial eclipse phase, but plateaued

by 24 hours post inoculation in the intracellular compartment at 2 x 10^3 TCID50 / ml and dropped off after 66 hours post-inoculation. Virus was not detected in the supernatant of infected HUH7 cells until 36 hours post inoculation and exhibited lower titers at all timepoints (Figure 4). Significant CPE was never observed in HUH7.0 cells. These results are consistent with previous report for both SARS-CoV and MERS-CoV suggesting similar replication dynamics between the zoonotic CoV strains (17, 18).

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 maintains a nucleotide sequence identical to that of the original US clinical strain. These deposits make 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 and frozen. Confirmed 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 antibiotic/antimyotic (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 (19, 20).

When CPE were observed, the cell monolayers were scrapped 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 using CDC's rRT-PCR assay and full genome sequencing (1) The CDC molecular diagnostic assay targets three portions of the N gene, and all three must be positive to be considered positive (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).

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), subsequently for Oxford Nanopore MinION sequencing. Consensus Nanopore sequences were generated using minimap 2.17 and samtools 1.9. Consensus sequences by Sanger sequences were generated from both directions using Sequencher 5.4.6, 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.) 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) MiniSeq instrument using the High-Output kit. Reads were processed with Trimmomatic v0.36 (21) to remove low quality base calls and any

adapter sequences. The *de novo* assembly program ABySS (22) 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. 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 (23) and visualized with the Integrated Genomics Viewer (24) 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* stained 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 (BioRAD) 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 rabbit polyclonal sera against the SARS-CoV spike (Sino Biological #40150-T52), β-Actin antibody (Cell Signaling Technology #4970), or a custom rabbit polyclonal sera against SARS-CoV nucleocapsid, 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 (25), 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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ACKNOWLEDGEMENTS

The reagent described is available through BEI Resources, NIAID, NIH: SARS-related coronavirus 2, Isolate USA-WA1/2020, NR-52281. We thank Dr. Mavanur R. Suresh for providing the plasmid, pBM302, expressing the SARS-CoV N protein. Research was supported by grants from NIA and NIAID of the NIH (U19AI100625 and R00AG049092 to VDM; R24AI120942 to SCW; AI99107 and AI114657 to SM). Research was also supported by STARs Award provided by the University of Texas System to VDM, funds from the Institute for Human Infections and Immunity at UTMB to SM, and trainee funding provided by the McLaughlin Fellowship Fund at UTMB

DISCLAIMERS

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention. Names of specific vendors, manufacturers, or products are included for public health and informational purposes; inclusion does not imply endorsement of the vendors, manufacturers, or products by the Centers for Disease Control and Prevention or the US Department of Health and Human Services.

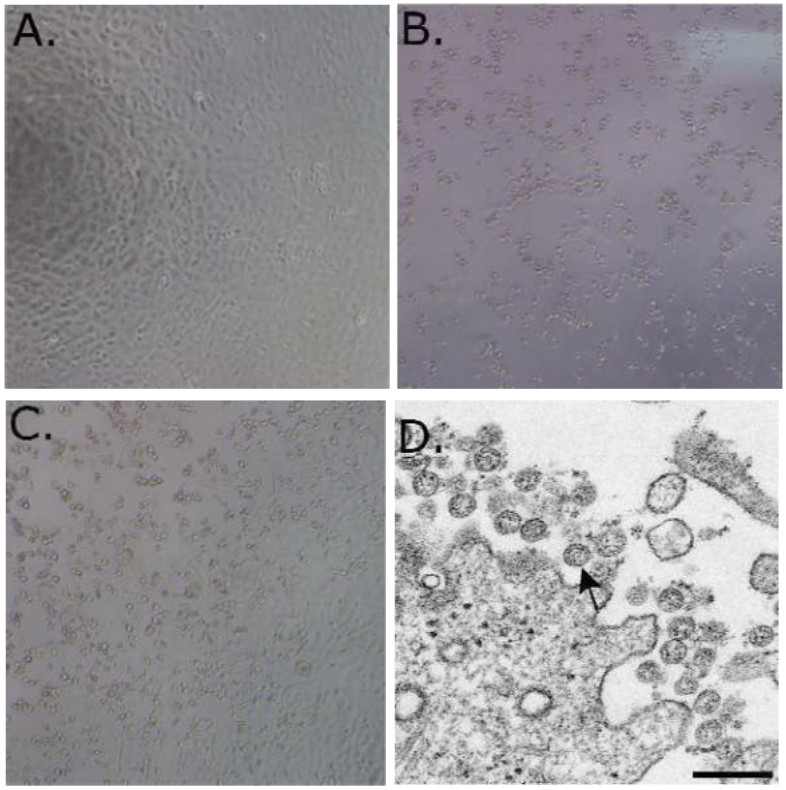

Figure 1.(A-C) 10X phase contrast images of vero monolayers at 3 days post-inoculation. Panels shown are (A) mock, (B) nasopharyngeal, and (C) oropharyngeal. (D) Electron microscopic image of the viral isolate showing extracellular spherical particles with cross sections through the nucleocapsids, seen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

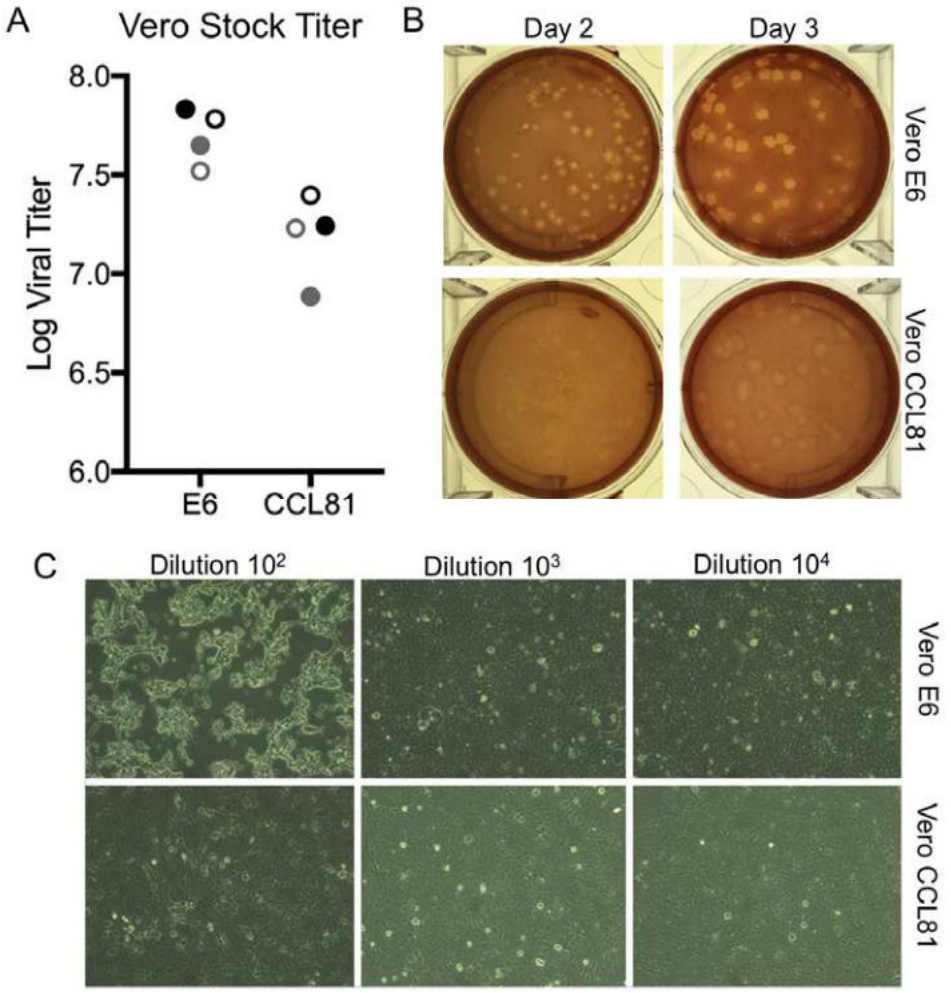

Figure 2. Viral propagation and quantitation. A) Two SARS-CoV-2 passage 4 stocks (black and gray) were quantified utilizing plaque assay at day two (closed circles) and day three (open circles) post infection of Vero E6 and Vero CCL81. B) Plaque morphology on Vero E6 and Vero CCL81 at day 2 and day 3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

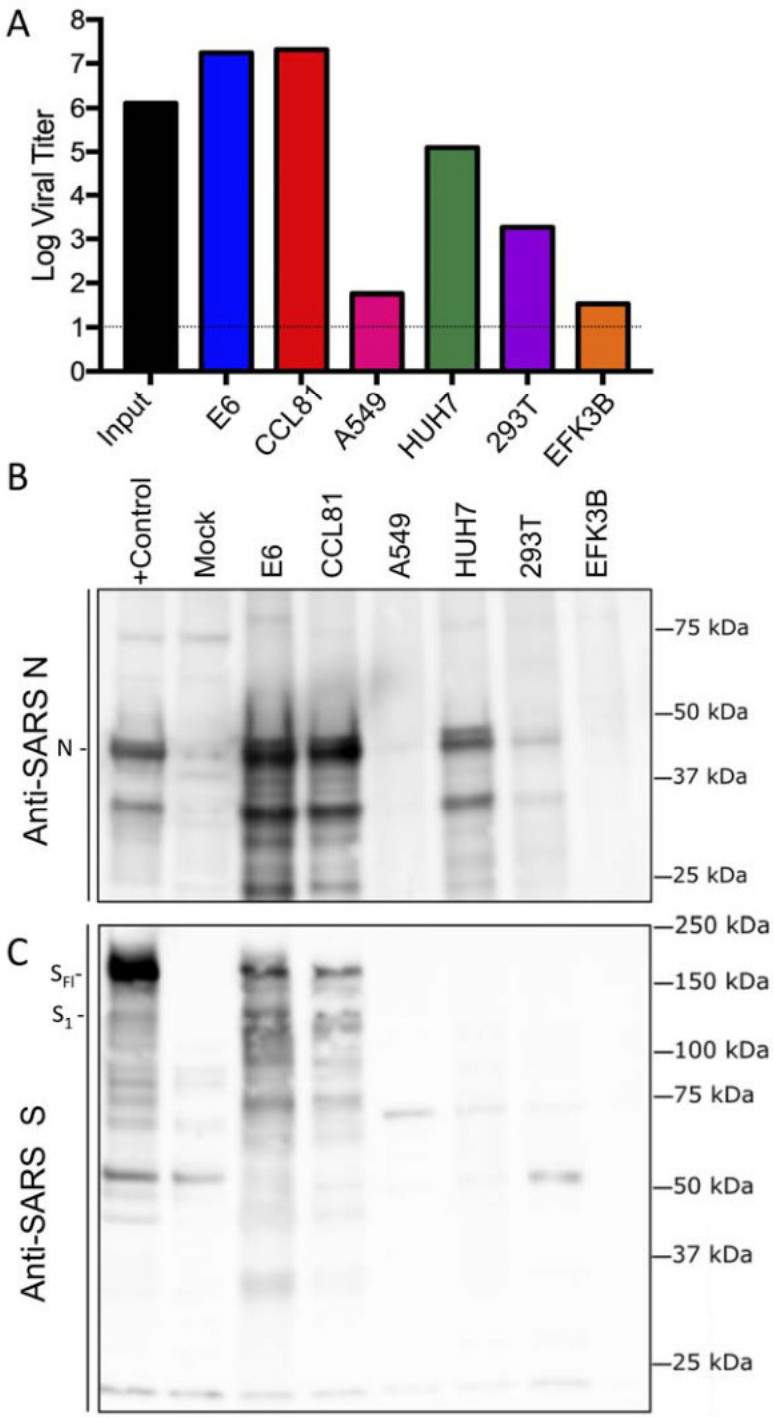

Figure 3. Cell lines susceptible to SARS-CoV-2. (A-C) Cell lines were infected with a high MOI of SARS-CoV-2 (MOI >5), washed after adsorption, and subsequently harvested 24 hours post infection for viral titer and protein lysates. A) Viral titer for SARS-CoV-2 was quantitated by plaque assay on Vero E6 cells two days post inoculation. B & C) Infected cell protein lysates were probed by western blot with B) rabbit polyclonal anti-SARS nucleocapsid (N) antibody or C) anti- SARS-CoV spike (S) antibody. N protein, full length spike (S_{FL}) and spike S1 (S_1) denoted.

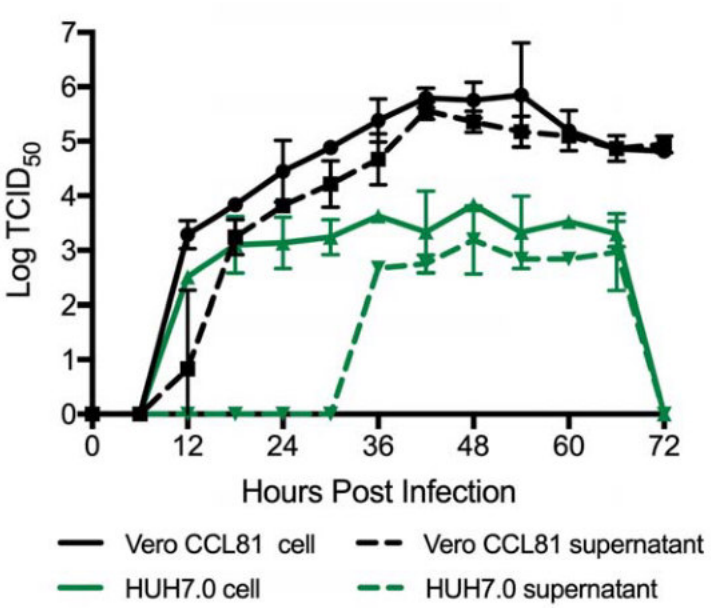

Figure 4. Multi-step growth curve for SARS-CoV-2. Vero CCL81 (Black) and HUH7.0 cells (green) were infected at MOI 0.1 and cells (solid line) and supernatants (dashed line) were harvested and assayed for viral replication via $TCID_{50}$.

Ministerio de Salud Pública

Dirección General de la Salud

Àrea de Vigilancia en Salud de la Población

División Epidemiología

Departamento de Laboratorios de Salud Pública

Montevideo, 28 de febrero de 2023

Pedido de Informe **Ref 2/001/3/8557/2022**

Se adjuntan referencias solicitadas

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Dra Rosario San Martín

Dir. Adjunta DLSP

Montevideo, 9 de Marzo de 2023

Ref. N° 12/001/3/1106/2023.-

Mediante acceso a la información pública se solicita:

“En una anterior consulta con REFERENCIA 12/001/3/8557/2022, que se me responde a la consulta ahí establecida sobre que el MSP TIENE conocimiento del CULTIVO del sarscov2 en otras instituciones y donde menciona a laboratorios del CDC de EE.UU, WUHAN EN China y Europeos; pregunto en esta oportunidad, y ya que tanto mi persona como profesionales médicos con más expertise que yo en búsquedas de este tipo y en estos laboratorios en las instituciones antes mencionadas, NO logramos obtener respuestas ni llegar a la información que desde la cartera aseguran que allí está, es que solicito de ser posible (y supongo que si ,porque manifiestan tener conocimiento) me brinden DIRECTAMENTE dicha información. Dicho de otra modo, me hagan el favor de repetir el procedimiento por el cual ustedes llegaron al conocimiento de esa información y facilitarmela con un link, sitio web o captura de pantalla con el acceso Directo y concreto para evacuar la la consulta realizada. Repito no tuvimos la capacidad de llegar a la información que debería estar al alcance entiendo.”

Consultada la Dirección General de la Salud, se adjunta informe que se sugiere notificar, y luce en los presentes obrados de fojas 7 a fojas 71.-

En virtud de los motivos expuestos, se sugiere hacer lugar a lo solicitado en los términos del presente informe.-

Ministerio de Salud Pública

Dirección General de Secretaría

VISTO: la solicitud de información pública efectuada, al amparo de lo dispuesto por la Ley Nº 18.381 de 17 de octubre de 2008;

RESULTANDO: I) que el peticionante solicitó oportunamente información acerca de si el Ministerio de Salud Pública, cuenta con información chequeada de quién tiene el virus SarsCoV2 cultivado, aislado, purificado y correctamente secuenciado acompañado de documentación para buscar la replicabilidad en laboratorios y comenzar los estudios adecuados;

II) que solicita una ampliación de la respuesta brindada por esta Secretaría de Estado a efectos de acceder a la información que no pudieron llegar;

CONSIDERANDO: I) que corresponde hacer lugar a lo peticionado;

II) que de acuerdo a lo dispuesto por el artículo 16 de la citada disposición legal, el acto que resuelva la petición debe emanar del jerarca máximo del Inciso o quien posea facultades delegadas al efecto;

ATENTO: a lo precedentemente expuesto y a lo establecido por Resolución Ministerial Nº 38/991 de 22 de enero de 1991;

EL DIRECTOR GENERAL DE SECRETARÍA

en ejercicio de las atribuciones delegadas

R E S U E L V E:

1º) Autorízase el acceso a la información, en referencia a la solicitud efectuada , al amparo de lo dispuesto por la Ley Nº 18.381, de 17 de octubre de 2008.

2º) Notifíquese a la parte interesada a través de Secretaría de la Dirección General de Secretaría. Pase al Departamento de Comunicaciones para su publicación en la página web institucional. Cumplido, archívese.

Ref. Nº 001-3-1106-2023

VC