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Supporting Information

Supplementary methods

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Appendix to: Caly L, Druce J, Roberts J, et al. Isolation and rapid sharing of the 2019 novel coronavirus (SAR-CoV-2) from the first patient diagnosed with COVID-19 in Australia. *Med J Aust* 2020; doi: 10.5694/mja2.50569.

Supplementary methods

1.1 Generation of SARS-CoV-2 cDNA

200µL aliquots from swab (nasopharyngeal in VTM), sputum, urine, faeces and serum samples were subjected to RNA extraction using the QIAamp 96 Virus QIAcube HT Kit (Qiagen, Hilden, Germany) and eluted in 60µL. Reverse transcription was performed using the BioLine SensiFAST cDNA kit (Bioline, London, United Kingdom), total reaction mixture (20µL), containing 10µL RNA extract, 4µL 5x TransAmp buffer, 1µL reverse transcriptase and 5µL nuclease-free water. The reactions were incubated at 25°C for 10 min, 42°C for 15 min and 85°C for 5 min.

1.2 Nested SARS-CoV-2 RT-PCR and Sanger sequencing

A PCR mixture containing 2µL cDNA, 1.6 µl 25mM $MgCl_2$, 4µL 10x Qiagen Taq Buffer, 0.4µL 20mM dNTPs, 0.3µL Taq polymerase (Qiagen, Hilden, Germany) and 2µL of 10 µM primer pools as described[2]. Briefly, first round included the forward (5'-GGKTGGGAYTAYCCKAARTG-3') and reverse (5'-GGKTGGGAYTAYCCKAARTG-3') primers. Cycling conditions were 94°C for 10min, followed by 30 cycles of 94°C for 30s, 48°C for 30s and 72°C for 40s, with a final extension of 72°C for 10 min. 2µL of resultant product was used as template for the second round which included the forward (5'-GGTTGGGACTATCCTAAGTGTGA-3') and reverse (5'-CCATCATCAGATAGAATCATCAT-3') primers. Cycling conditions were 94°C for 10min, followed by 40 cycles of 94°C for 30s, 50°C for 30s and 72°C for 40s, with a final extension of 72°C for 10min. Resultant products had an expected size of approximately 440bp on a 2% agarose gel. The PCR products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) and sequenced using an Applied Biosystems SeqStudio Genetic Analyzer (Life Technologies, Carlsbad, CA, USA) using Big Dye Terminator 3.1 (Life Technologies, Carlsbad, CA, USA) and Round 2 PCR primers above. SARS-CoV and 229e-CoV cDNA were used as positive control material.

1.3 Detection of SARS-CoV-2 using an In-house TaqMan Real-time RT-PCR assay.

TaqMan RT-PCR assay comprised of 2.5 µl cDNA, 10 µl Primer Design PrecisonPLUS qPCR Master Mix 1 µM Forward (5'–AAA TTC TAT GGT GGT TGG CAC AAC ATG TT-3'), 1 µM Reverse (5'- TAG GCA TAG CTC TRT CAC AYT T–3') primers and 0.2 µM Probe (5'–FAM- TGG GTT GGG ATT ATC-MGBNFQ–3') targeting the BetaCoV family RdRP (RNA Dependent RNA Polymerase) gene. The real-time RT-PCR assay was performed on an Applied Biosystems ABI 7500 Fast Real-time PCR machine (Applied Biosystems, Foster City, CA, USA) with cycling conditions 95°C for 2 min, 95°C for 5 s, 60°C for 24 s. SARS-CoV cDNA (Ct~30) was used as a positive control.

2.1 Cell culture of SARS-CoV-2 and electron microscopy

Vero/hSLAM cells (African green monkey kidney cells transfected to express the human signaling lymphocytic activation molecule (SLAM; also known as CDw150)[1] were grown at 37°C, 5% CO_2 in maintenance media consisting of 10mL Earle's minimum essential medium (EMEM), 7% fetal bovine serum (FBS) (Bovogen Biologicals, Keilor East, AUS) 2mM L-glutamine, 1 mM sodium pyruvate, 1500mg/L sodium bicarbonate, 15 mM HEPES and 0.4mg/ml geneticin to 95% confluency in 25cm^2 flasks. Prior to use for isolation, maintenance media was removed from the flask and 500µL of respiratory swab inoculum was overlaid on the cell monolayer. The flask was returned to the 37°C incubator to allow the virus to adsorb for 1 hour before addition of 10 mL viral culture media (EMEM as above but FBS reduced to 2%). Flasks were monitored for viral cytopathic effect and 140µL aliquots of supernatant removed every 48 hours to assess virus burden by TaqMan real-time RT-PCR. First passage culture grown virus isolate was subsequently shipped nationally and internationally in packaging compliant with UN 2814 Category A shipping requirements using credentialed, specialised courier services under the appropriate Australian government export approvals processes and receiving country import permissions.

2.2 Electron microscopy

Negative staining and thin sectioning of cell culture-derived supernatant was performed after low speed centrifugation at 1000g for 3 minutes. Copper electron microscopy grids coated with formvar and a continuous carbon layer were glow-discharged for 30 seconds using a Pelco EasiGlow system. 8μL of supernatant was applied directly to a glow-discharged grid and allowed to adsorb for 20 seconds, then negative stained with 8μL of 3% phosphotungstic acid (pH 7.0). The negative-stained grid was air-dried at room temperature and examined using an FEI T12 Spirit electron microscope operating at an acceleration voltage of 80keV. Electron micrographs were collected using an FEI Eagle 16MP CCD camera. Virion diameter and glycoprotein features were measured using the FEI TIA software package. After low speed centrifugation, the supernatant was removed and the remaining pellet was enrobed in 3% agarose, after fixation in 2% osmium tetroxide the sections were passed through a gradient series of dehydrating ethanol solutions and then transitioned to 100% propylene oxide for embedding in Spurr's resin. Embedded samples were polymerised at 60ºC for 72 hours then thin-sectioned using a Leica UC7 ultramicrotome, 70nm sections were transferred to copper 100-mesh electron microscopy grids coated with formvar and a continuous carbon layer and examined using an FEI F30 Twin electron microscope operating at an acceleration voltage of 200keV. Electron micrographs were collected using an FEI Ceta 16MP CCD camera.

3.1 RNA extraction and whole genome sequencing

RNA extraction and whole genome sequencing (WGS) of the viral isolate was performed at the Victorian Infectious Diseases Reference Laboratory (VIDRL), at the Doherty Institute, University of Melbourne. RNA was extracted from clarified (6000g x 10min, 4°C) cell culture supernatant using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). Carrier RNA was not added to buffer AVL with 1% linear acrylamide (Life Technologies, Carlsbad, CA, USA) used instead. Wash buffer AW1 was omitted from the purification stage with RNA eluted in 50μL of nuclease free water, followed by DNase treatment with Turbo DNase (Thermo Fisher Scientific, Waltham, MA, USA)

37°C for 30min. RNA was cleaned and concentrated to 8μL using the RNA Clean & Concentrator-5 kit (Zymo Research, Irvine, CA, USA), as per manufacturer's instructions. Randomly amplified cDNA was prepared using the sequence-independent single-primer amplification (SISPA) approach,[3,4] For reverse transcription, 4 μL RNA and 1μL SISPA primer A (5'–GTTTCCCACTGGAGGATA-N9–3', 40 pmol/μL) were combined and incubated for 5min at 65°C and then cooled to 4°C for 5min. First-strand synthesis was performed by adding 2μL SuperScript IV first-strand buffer, 1μL 10mM dinucleoside triphosphates (dNTPs), 0.5μL 0.1M dithiothreitol (DTT), 1μL nuclease-free water and 0.5μL SuperScript IV (Thermo Fisher Scientific, Waltham, MA, USA) before incubation at 50°C for 10min. Second-strand synthesis was performed by adding 1μL 5x Sequenase buffer, 3.85μL nuclease-free water, and 0.15μL sequenase (Affymetrix, Santa Clara, CA, USA) prior to incubation for 8min at 37°C, followed by the addition of 0.45μL sequenase dilution buffer and 0.15μL sequenase and incubated 37°C for 8min again. Amplification of cDNA was performed using 5μL reaction mixture as input to a 50μL AccuTaq LA (Sigma-Aldrich, St. Louis, MO, USA) reaction mixture with 5μL AccuTaq LA 10x Buffer, 2.5μL 10mM dNTP mix, 1μL DMSO, 0.5μL AccuTaq LA DNA polymerase, 35μL nuclease-free water and 1 μL SISPA primer B (5'–GTTTCCCACTGGAGGATA–3'), with PCR cycling conditions of 98°C for 30s, 30 cycles of 94°C for 15s, 50°C for 20s, and 68°C for 2min, followed by 68°C for 10min. Amplified cDNA purified using AMPure XP beads (Beckman Coulter, Brea, CA) at a 1:1 ratio (ie, 50μL beads to 50μL reaction volume) and eluted in 48μL nuclease-free water. cDNA was quantified using a Qubit high-sensitivity double-stranded DNA (dsDNA) kit (Thermo Fisher Scientific, Waltham, MA, USA), according to manufacturers' instructions.

3.2 Nanopore library preparation and sequencing.

Sequencing libraries were prepared using 300ng cDNA as input for the SQK-LSK109 kit (Oxford Nanopore Technologies, Oxford, UK) as per manufacturer's instructions. Libraries were sequenced on FLO-MIN106 flow cells on the MinION Mk1b device (Oxford Nanopore Technologies, Oxford,

UK), with sequencing for 30h. Basecalling was performed using Guppy v3.4.5 GPU with the high-accuracy dna_r9.4.1_450bps_hac config file on an Nvidia GTX1080 graphics card on a Ubuntu 16.0.4 workstation. SISPA barcode sequences were removed using the "Trim Ends" command in Geneious Prime (version 2020.0.5), and the Minimap2 v2.17 plugin used to align the reads to NCBI reference sequence MN908947.3.

3.3 Illumina library preparation and sequencing

An Illumina (Illumina Inc., San Diego, California, USA) library was prepared using a Nextera XT kit, using 75b paired-end chemistry, and were sequenced on an Illumina MiSeq platform as per manufacturer's instructions. Approximately 30,000,000 reads were generated from the SISPA input material.

3.4 Phylogenetic analysis and genome assembly

The assembly of the SARS-CoV-2/VIC01/Australia genome (GenBank Accession MT007544.1, GISAID EPI_ISL_406844) was confirmed using both *de novo* assembly and alignment to a reference. *De novo* assembly of Illumina reads used Shovill v1.0.9 (https://github.com/tseemann/shovill). Reference guided assembly was performed by alignment of Illumina reads to the Wuhan-Hu-1 reference genome (MN908947.3) using Snippy 4.4.6 (https://github.com/tseemann/snippy) and Geneious Prime 2020.0.0, and alignment of Nanopore reads using Minimap2.[5] The complete BetaCoV/Australia/VIC01/2020 genome demonstrated 3 single nucleotide polymorphisms (SNPs) compared to the Wuhan-Hu-1 reference genome (MN908947.3), with 2 SNPs having a 10% minor wildtype allele frequency. Further, a 10 bp deletion was detected in the 3' UTR, not previously described.

References

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