Isolation and rapid sharing of the 2019 novel coronavirus (SAR-CoV-2) from the first diagnosis of COVID-19 in Australia

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ABSTRACT

Objective(s):

The isolation and rapid sharing of the 2019 novel coronavirus (SARS-CoV-2) is critical to the effective control of this new outbreak through the development of better diagnostics and potential vaccine candidates and antiviral agents.

Design, setting, participants:

A 58 year old male from Wuhan, China arrived in Melbourne, Australia on 19th January 2020 and was admitted to hospital via the emergency department on 24th January 2020 with symptoms of fever, cough, and progressive dyspnea. This report describes the clinical course and laboratory features of the first reported case of COVID-19 (the illness caused by SARS-CoV-2) in Australia, along with isolation, whole genome sequencing, imaging and rapid sharing of virus.

Results:

A nasopharyngeal swab and sputum taken on presentation tested positive for SARS-CoV-2 using RT-PCR. Inoculation of Vero/hSLAM cells with material from the initial nasopharyngeal swab led to the isolation of SARS-CoV-2 virus in culture. Electron microscopy of the supernatant confirmed the presence of virus particles displaying morphology characteristic of the family *Coronaviridae*. Whole genome sequencing of the viral isolate and phylogenetic analysis revealed that the genome sequence from this patient exhibited >99.99% sequence identity to other publicly available SARS-CoV-2 genomes. Within 24 hours of isolation the first Australian viral isolate of SARS-CoV-2 was shared with domestic and international reference laboratories and major North American and European culture collections.

Conclusion(s):

The ability to rapidly identify, propagate and share the SARS-CoV-2 isolate globally represents an important step in collaborative scientific efforts in response to this public health emergency.

INTRODUCTION

Since the first identification of an outbreak of the 2019 novel coronavirus (SARS-CoV-2) respiratory infections in Wuhan, China, the scale and tempo of the global response has been unparalleled.[1-6] In particular, the rapid sharing and integration of clinical and epidemiological data has facilitated understanding of the spectrum of clinical disease caused by SARS-CoV-2, and the extent of global spread, although there are still many unanswered questions. Further, rapid genomic analyses have corroborated epidemiological investigations suggesting a global point-source outbreak, originating in Wuhan, China, caused by a novel betacoronavirus.[2, 5] Fundamental pillars to the control of any infectious disease, including this current outbreak, are effective prevention, diagnostic and treatment strategies. For viral pathogens, one of the most important developments in facilitating these steps is propagation of live virus, and subsequent timely dissemination of the viral isolate to scientific and public health agencies both domestically and internationally. This rapid sharing of material allowed laboratories to validate their diagnostic assays and confirm their ability to detect SARS-CoV-2. This report describes the clinical course and laboratory features of the first reported case of COVID-19 (the illness caused by SARS-CoV-2) in Australia, along with isolation, sequencing and imaging of the virus.

Case report and clinical course

A 58 year old male from Wuhan, China felt unwell on the day of arrival in Melbourne, Australia on 19th January 2020. He had no contact with live food markets, known cases of COVID-19 or hospitals. His past medical history included Type 2 diabetes mellitus, and he ceased smoking four years previously. He developed fever on the 20th January 2020, a cough with sputum production on the 23rd January 2020, and was admitted to hospital via the emergency department on 24th January 2020 with progressive dyspnea. Examination revealed

a temperature of 38.1°C, heart rate 95 beats/min and O_2 saturation 94% on room air. A chest radiograph demonstrated subtle ill-defined opacities within the mid zones bilaterally, and in the left lower zone. A thoracic CT scan performed on day four of admission revealed extensive ground-glass opacities in a peribronchovascular and peripheral distribution, favouring the mid to upper zones of the lungs (Figure 1). Full blood examination demonstrated lymphocytes 0.80 x 10^9/L (normal range 1.0-4.0 x 109/L). C reactive protein peaked on day six of admission at 182 mg/L (0-5 mg/L). Liver function test abnormalities peaked on day 12 of admission: alkaline phosphatase 210 U/L (30-110 U/L), gamma glutamyl transferase 416 U/L (30-110 U/L), alanine aminotransferase 183 U/L (5-40 U/L), and a liver ultrasound revealed hepatic steatosis. Intravenous ceftriaxone and azithromycin were commenced on day four for possible secondary bacterial pneumonia, although no bacterial pathogen was identified. Low-flow oxygen (maximum 3L/min via nasal prongs) was administered until day 10 of admission. He made gradual clinical improvement, with resolution of fever, productive cough and dyspnea by day 12 of admission, and was discharged from hospital on the 7th February, 2020.

METHODS

Diagnostic testing for SARS-CoV-2

Real time reverse transcription (RT)- polymerase chain reaction (PCR) for SARS-CoV-2 was performed using 200 μL of viral transport media (VTM) collected from an initial nasopharyngeal swab and separately from sputum, urine, faeces and serum (Supplementary Appendix). Briefly, an in-house real-time RT-PCR assay was developed with all positive tests further confirmed by a nested RT-PCR, as previously described. [7] PCR products underwent in-house Sanger sequencing, which confirmed the presence of SARS-CoV-2.

Viral culture and electron microscopy

Material from the initial nasopharyngeal swab was used to inoculate a Vero/hSLAM cell line (Supplementary Appendix). Flasks were monitored for the formation of viral cytopathic effect (CPE) and 140 µl aliquots removed every 48 hours to assess virus burden by real-time RT-PCR.

For electron microscopy, a 4 mL aliquot of cell culture supernatant grown in the presence of 4µg/ml trypsin was inactivated using 0.5% glutaraldehyde for 12 h, then clarified by centrifugation at 1,000 x g for 3 min. Supernatant was negatively stained using 3% phosphotungstic acid at pH 7.0, then examined using an FEI Technai T12 electron microscope at 80kv. The remaining pellet was stained en-bloc and embedded in resin, 70nm sections were examined using a FEI F30 electron microscope at 200kv.

Sequencing of SARS-CoV-2 and bioinformatic analysis

RNA extraction and whole genome sequencing (WGS) of the viral isolate was performed (Supplementary Appendix). Briefly, RNA was extracted from clarified cell culture supernatant, and randomly amplified cDNA was prepared using the Sequence-Independent Single-Primer Amplification (SISPA) approach.[8, 9] Sequencing was performed using a combination of Oxford Nanopore Technologies (ONT) and Illumina short-read sequencing (Supplementary Appendix). Genomic assembly of the BetaCoV/Australia/ VIC/01/2020 genome was confirmed through parallel *de novo* and reference guided methods (Supplementary Appendix).

RESULTS

Detection of SARS-CoV-2 in multiple clinical samples

A nasopharyngeal swab and sputum taken on presentation tested positive for SARS-CoV-2 using a real-time RT-PCR assay (Supplementary Appendix). Serial daily RT-PCR testing of nasopharyngeal swabs and sputum taken from this patient (Figure 2) revealed a gradual decline in viral load in sputum between day one and day eight post-admission, and a decline in viral load and disappearance from nasopharyngeal swabs by day seven. No virus was detected in regular urine samples, nor in a single faecal sample on day three, nor in plasma on day one.

Growth, visualisation and global sharing of SARS-CoV-2 virus

Two-days post-inoculation of the VERO-hSLAM cell line, subtle viral CPE was observed, becoming distinct at day six (Figure 3A) compared to an uninfected control cell line (Figure 3B). RT-PCR testing of the cell line supernatant confirmed a high viral load, suggestive of productive viral infection (Figure 3C). Electron micrographs of the negative-stained supernatant indicated the presence of spherical and pleomorphic virus-like particles measuring 90 to 110 nm. The virus particles displayed prominent spikes measuring 9 to 12 nm, characteristic of viruses belonging to the family *Coronaviridae* (Figure 3D-i). Electron micrographs of sectioned VERO-hSLAM cells showed cytoplasmic membrane-bound vesicles containing coronavirus particles (Figure 3D-ii) After multiple failures to recover virions with the characteristic fringe of surface spike proteins, it was found that the addition of trypsin to the cell culture media immediately improved virion morphology.

In consultation with the World Health Organisation (WHO) the viral isolate was shared with domestic and international reference laboratories within 24 hours, and lodgement with major North American and European culture collections for onward distribution is currently underway.

Phylogenetic analysis

Phylogenetic analysis revealed that the genome sequence (BetaCoV/Australia/VIC01/2020) from this patient exhibited >99.99% sequence identity to other publicly available SARS-CoV-2 genomes (Supplementary Appendix), consistent with the epidemiological features of this case originating in Wuhan.[10] Compared to the SARS-CoV-2 reference sequence, (NC_045512.3), three previously described single nucleotide polymorphisms (SNPs) were detected, and a 10bp deletion in the 3'UTR (Supplementary Appendix). Sequences are available at GenBank Accession MT007544.1, and the genome was rapidly uploaded to the Global Initiative of Sharing All Influenza (GISAID) (EPI_ISL_406844).

DISCUSSION

Here, we describe the first reported case of COVID-19 in Australia, with rapid diagnosis, isolation, imaging and sharing of the causative agent, SARS-CoV-2. As of 4th March 2020, there are an additional 41 cases in Australia. Of these 15 cases had direct or indirect travel history to Wuhan, China, 10 are associated with the Diamond Princess repatriation from Japan, 10 have reported to have direct or indirect travel history to Iran and 4 have reported travel history to Singapore, United Arab Emirates, Japan and South Korea. Two cases have no reported travel history to high risk countries and are currently under investigation. Although the vast majority of cases are within China, there is an increasing number of cases

within South Korea, Italy and Iran , and reports describing limited human to human transmission are emerging[6,10,11]. Although case numbers in Australia are relatively small, the political and societal effects in Australia (as in other countries) have already been considerable, with travel restrictions to and from mainland China and Iran .[12] The sustainability of these measures, and the effects on local and global control remain to be seen, but it is likely that the consequences of this outbreak will continue to be felt for many months, if not longer.

Clinical features of the case described here are in keeping with other emerging reports, with an initial presentation of fever, cough, and progressive dyspnea.[3, 6] Of note, in this patient, sputum specimens had the highest burden of virus and remained positive for SARS-CoV-2 longer (up to eight days post presentation) compared to four days post presentation for nasopharyngeal swabs (Figure 2). A decrease in viral load was noted to correlate with the resolution of fever, and ultimately, with clinical improvement. One unresolved question from current clinical studies is whether patients who are clinically stable and deemed fit to be discharged from hospital, who possess detectable virus by PCR are infectious, or whether this represents persistence of non-infectious residual viral RNA.

Although we used a standard approach to virus isolation, we were the first to isolate the virus during the early stages of the epidemic outside of China. Potential reasons for this success could be related to the viral burden of the specimen, or simply our long standing clinical experience within our reference laboratory

To date, an important scientific response during this outbreak response has been rapid sharing of information on diagnostic assays and genomic data, enabling a real-time picture of the emergence and spread of this novel virus. In addition to these aspects, a major principle of our laboratory response in Australia was to immediately share the viral isolate with the WHO and other laboratories, allowing for rapid diagnostic test validation to occur. We are continuing to share live virus with other agencies, both domestically and internationally, involved in the development and testing of therapeutic agents and vaccines. We believe this is an essential function of public health reference as well as research laboratories, and strongly encourage others to use a similar collaborative approach to streamline efforts to diagnose, prevent and treat cases during this public health emergency.

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SUPPLEMENTARY APPENDIX

ACKNOWLEDGMENTS

[Blinded for submission]

FUNDING

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SUPPLEMENTARY METHODS

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 10 mL Earle's Minimum Essential Medium (EMEM), 7% Fetal Bovine Serum (FBS) (Bovogen Biologicals, Keilor East, AUS) 2 mM 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 reduced FBS to 2%). Flasks were monitored for the formation of CPE and 140 µl 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.

Electron microscopy

Negative-staining and thin-sectioning of cell culture-derived supernatant was performed after low speed centrifugation at 1,000g 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. 8 µL of supernatant was applied directly to a glow-discharged grid and allowed to adsorb for 20 seconds, then negative stained with 8uL 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 80 keV. 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 200 keV. Electron micrographs were collected using an FEI Ceta 16MP CCD camera.

Generation of SARS-CoV-2 cDNA

200 µL 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 60 µl. Reverse transcription was performed using the BioLine SensiFAST cDNA kit (Bioline, London, United Kingdom), total reaction mixture (20 µl), containing 10 µL of RNA extract, 4 µl of 5x TransAmp buffer, 1µl of Reverse Transcriptase and 5 µl of Nuclease free water. The reactions were incubated at 25°C for 10 min, 42°C for 15 min and 85°C for 5 min.

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

A PCR mixture containing 2 µl cDNA, 1.6 µl of 25 mM $MgCl_2$, 4 µl of 10x Qiagen Taq Buffer, 0.4 µl of 20 mM dNTPs, 0.3 µl of Taq polymerase (Qiagen, Hilden, Germany) and 2 µl 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 10 min, followed by 30 cycles of 94°C for 30 s, 48°C for 30 s and 72°C for 40 s, 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 10 min, followed by 40 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 40 s, with a final extension of 72°C for 10 min. 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.

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.

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 10 min, 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 50 µl of nuclease free water, followed by DNase treatment with Turbo DNase (Thermo Fisher Scientific, Waltham, MA, USA)

37°C for 30 min. RNA was cleaned and concentrated to 8 µl 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 of RNA and 1 µl of SISPA primer A (5'-GTTTCCCACTGGAGGATA-N9-3', 40 pmol/µl) were combined and incubated for 5 min at 65°C and then cooled to 4°C for 5 min. First-strand synthesis was performed by adding 2 µl of SuperScript IV first-strand buffer, 1 µl of 10 mM dinucleoside triphosphates (dNTPs), 0.5 µl of 0.1 M dithiothreitol (DTT), 1 µl of nuclease free water and 0.5 µl of SuperScript IV (Thermo Fisher Scientific, Waltham, MA, USA) before incubation at 50°C for 10 min. Second-strand synthesis was performed by adding 1 µl of 5x Sequenase buffer, 3.85 µl nuclease free water, and 0.15 µl of Sequenase (Affymetrix, Santa Clara, CA, USA) prior to incubation for 8 min at 37°C, followed by the addition of 0.45 µl Sequenase dilution buffer and 0.15 µl of Sequenase and incubated 37°C for 8 min again. Amplification of cDNA was performed using 5 µl of the reaction mixture as input to a 50 µl AccuTaq LA (Sigma-Aldrich, St. Louis, MO, USA) reaction mixture with 5 µl of AccuTaq LA 10x Buffer, 2.5 µl of 10 mM dNTP mix, 1 µl of DMSO, 0.5 µl of AccuTaq LA DNA Polymerase, 35 µl of nuclease free water and 1 µl of SISPA primer B (5'-GTTTCCCACTGGAGGATA-3'), with PCR cycling conditions of 98°C for 30 s, 30 cycles of 94°C for 15 s, 50°C for 20 s, and 68°C for 2 min, followed by 68°C for 10 min. Amplified cDNA purified using AMPure XP beads (Beckman Coulter, Brea, CA) at a 1:1 ratio (ie. 50 µl beads to 50 µl reaction volume and eluted in 48 µl 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.

Nanopore library preparation and sequencing.

Sequencing libraries were prepared using 300 ng of 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 30 h. 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.

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.

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.

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Figures

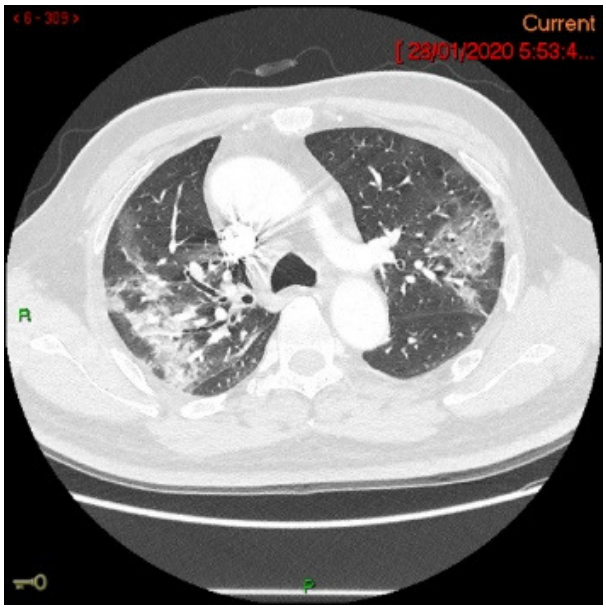

Figure 1. Thoracic CT scan performed day four post presentation**.**

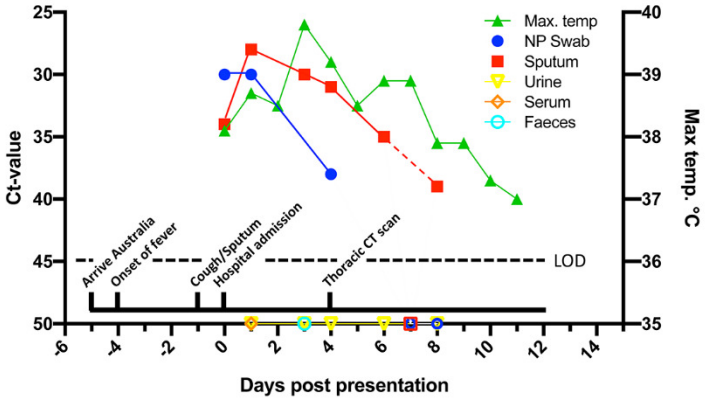

Figure 2. Clinical course with associated laboratory investigations. Clinical events and temperature (green) are shown over the time of admission to hospital. SARS-CoV-2 was quantified by real-time RT-PCR and the cycle threshold (Ct) shown for each of the nasopharyngeal swab (blue), sputum (red), urine (yellow), serum (orange) and faeces (cyan).

An increase in Ct value is consistent with a decrease in viral load. The assay limit of detection (LOD) threshold is Ct=45. Open symbols below this threshold demonstrate no detection of virus.

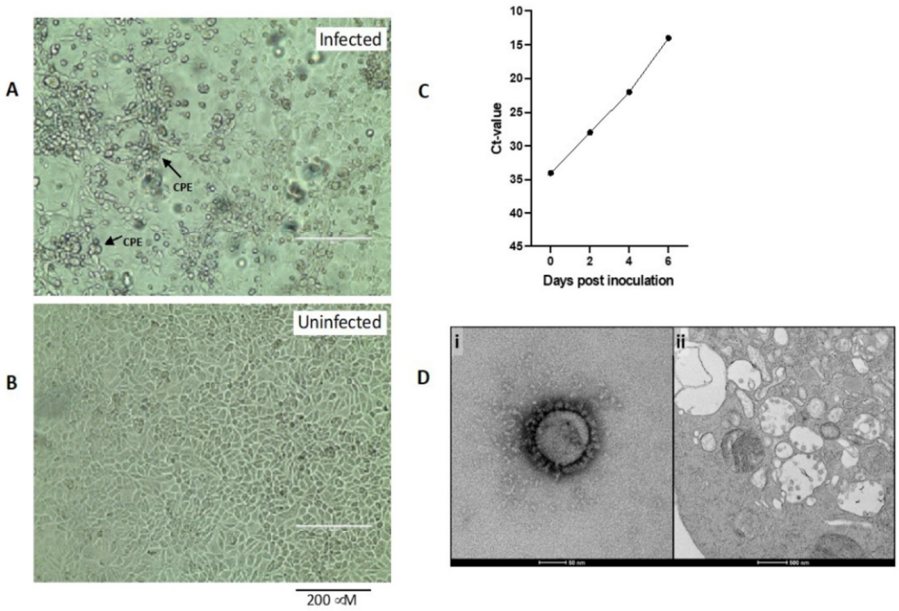

Figure 3. (A) Light microscopy of Vero/hSLAM cells which were (A) infected with patient material showing viral cytopathic effect (CPE) six days post-inoculation or (B) not infected. (C) Quantification of viral load using real-time PCT of supernatant collected from infected Vero/hSLAM cells. Lower threshold cycles (Ct values) represent a higher viral load. (D) Electron micrographs showing spherical and pleomorphic viral particles of mean size 100 nm displaying the characteristic crown-like fringe of spike proteins and infected VERO/hSLAM sections with membrane-bound vesicles containing virus.