Adjunctive viral cell culture supports treatment decision making in patients with secondary humoral immunodeficiency and persistent SARS-CoV-2 infection

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Short title

SARS-CoV-2 viral cell culture support of haem-oncology decisions

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Persistent Severe Acute Respiratory Syndrome coronavirus-2 (SARS-CoV-2) infection is observed among patients with haematological malignancy, conferring an increased mortality risk.[1-2] Persistent SARS-CoV-2 RNA detection from clinical samples may represent redundant fragmented RNA, replication-competent virus, or reinfection.[3-4]

Given the role of the host immune response in viral clearance and COVID-19 immunopathogenesis,[5-6] distinguishing these scenarios is important for therapeutic decision-making (antiviral vs immunomodulatory) as well as preventing onward hospital transmission. Optimal timing of subsequent chemotherapy cycles is challenging, since relapse of COVID-19 can occur in individuals with impaired humoral responses.[7]

We present the investigation of relapsing SARS-CoV-2 pneumonitis, with virological persistence evidenced by SARS-CoV-2 cell culture and sequencing, in the context of cellular and humoral immunodeficiency secondary to underlying lymphoma and chemo-immunotherapy. The potential clinical benefit of cell culture is discussed.

Investigation

The patient was investigated for persisting SARS-CoV-2 infection/reinfection following relapsing symptomatic pneumonitis associated with positive RNA-polymerase chain reaction (PCR) testing after second-round chemotherapy. Investigation was conducted as per current Public Health England guidelines, including infection specialist advice, inhouse whole genome sequencing (WGS) and immunological testing using both B cell and T cell assays (**Supplementary Information**).[8,9] Additionally, inhouse viral cell culture was conducted to assess for replication-competent virus (**Supplementary Information**).

Case

A 65-year-old lady who underwent renal transplant in 1995 for focal segmental glomerulonephritis, followed by long term immunosuppression with tacrolimus, subsequently developed low-level lymphocytosis in 2017. Immunophenotyping confirmed a B-cell non-Hodgkin lymphoma (NHL), histologically most likely to be diffuse large B cell lymphoma, associated with an IgM paraprotein of 6g/L for which she was managed expectantly.

In November 2020, she presented acutely with abdominal pain. Computed tomography imaging demonstrated a bulky retroperitoneal soft tissue mass at the porta hepatis, associated with 4kg weight loss. Bone marrow aspirate demonstrated infiltration with a

CD19+ve, CD5-ve B cell NHL. Gastroscopy showed abnormal appearance of gastric folds and gastric biopsy confirmed CD5+ve stage IVB high grade B-cell NHL.

On 7 December 2020, four days post first-cycle R-CHOP (rituximab/cyclophosphamide/doxorubicin/vincristine and prednisolone) chemotherapy, our patient tested PCR-positive for SARS-CoV-2 infection associated with cough, fever, and shortness of breath without oxygen requirement. Ongoing symptomatic and PCR-positive SARS-CoV-2 infection with cough/dyspnoea (day 21) delayed a second cycle. Due to the lack of oxygen requirement at this time, neither remdesivir nor dexamethasone were administered. On day 52, once symptoms had fully resolved and a SARS-CoV-2 PCR was negative, she received a delayed second cycle of chemotherapy. Neutropenia ($0.0x10^9$/L) and fever (38^oC) subsequently developed (day 58) (**Figure 1A, 1B**). Lymphocyte count was $0.0x10^9$/L, remaining $<1.0x10^9$ for 38 days post-chemotherapy. Further SARS-CoV-2 PCR tests were positive on days 58/59/63/67 (same B.1.77.5 lineage, **Figure 2**). Six doses of G-CSF were administered on days 58-60, 62-64 (neutrophil nadir 0.0, peak $6.6x10^9$/L). Tacrolimus levels remained within range. New bilateral pulmonary infiltrates on chest imaging (**Figure 1**), C-reactive protein (CRP) of 326mg/L and rising neutrophil count followed (day 66), alongside new oxygen requirements. Investigation for possible coinfection included urinary, sputum, serial blood cultures as well as carrying out PCR on ethylenediaminetetra-acetic acidblood for cytomegalovirus and adenovirus. Atypical urinary antigens and a syndromic respiratory PCR panel (Biofire FilmArray Respiratory Panel 2.1, Biofire Diagnostics, bioMerieux, Salt Lake City, Utah, USA) targeting 22 virus and bacteria targets were also conducted. All results were negative. Increased prednisolone (40mg daily, tapering 10mg daily every 5 days) and remdesivir (200mg stat, 100mg daily for 5 days) were commenced (day 67): fever, dyspnoea and oxygen requirement resolved, and CRP declined to 22mg/L (**Figure 1b**).

PCR tests remained positive on days 76 (decreased cycle threshold number (Ct) value), 83, 86 and 91, becoming negative from day 134 onwards (**Figure 1a**). Viral cell culture performed on day 64 and 86 (before and after remdesivir, with both swabs also PCR-positive) demonstrated replication-competent virus (approximately 400-fold increase of detectable RNA over inoculum [**Figure 1]**). WGS of viral RNA showed ongoing infection with the same B.1.177.5 lineage throughout (**Figure 2**), acquiring 3 single nucleotide polymorphisms (SNPs) between day 1-63 and a further 5 SNPs between days 64-86 (after remdesivir).

SARS-CoV-2 specific anti-nucleocapsid and anti-S antibodies were negative, whereas enzyme-linked immunoabsorbent spot (ELISpot) readout was positive (>160 spots across the S1/S2 panels) indicating robust T-cell responses to a panel of SARS-CoV-2 specific peptide pools.

Discussion

Determining SARS-CoV-2 viability in patients with haematological malignancy has implications for treatment-based decision-making. For example, B-cell depletion with rituximab should ideally be held during viable SARS-CoV-2 infection, to avoid hindering antibody responses. In this case, viral cell culture results alongside WGS supported reactivation of symptomatic infection and led to the decision to treat with a full course of high-dose steroids and remdesivir. Moreover, administration of G-CSF therapy for supportive management of chemotherapy-induced neutropenia was highlighted as a possible risk for further symptomatic (fever, breathlessness) disease in the presence of infection with replication-competent SARS-CoV-2 virus. Recognition of replication-competent virus prior to administration of G-CSF may therefore allow improved patient understanding of risk and advanced planning should pneumonitis develop.

In addition to stimulating neutrophil proliferation and maturation, G-CSF reconstitutes immune mediators including pro-inflammatory cytokines interleukin-1, TNF-α and interleukin-6,[6] which play a role in a maladaptive inflammatory response to SARS-CoV-2 infection.[10] Furthermore, autopsy studies reveal aggregated neutrophils and neutrophil extracellular traps in lung tissue,[11] induced by a mechanism that appears dependent on active SARS-CoV-2 viral replication.[10] We therefore postulate that rising neutrophils following G-CSF therapy during active SARS-CoV-2 infection may have led to relapsing pneumonitis in this manner, clinically resembling paradoxical immune reconstitution inflammatory syndromes (IRIS) (**Figure 1b**), occasionally seen with treatment initiation of HIV or tuberculosis,[12-13] with one such case reported.[14] As with IRIS, treatment with steroids may have contributed to resolution of CRP and oxygen requirement (**Figure 1**) although timing of G-CSF alongside steroids is a confounding factor that makes further interpretation in this case difficult.

A further consideration is the potential for ongoing infectivity despite remdesivir treatment and symptom resolution. Clinical and virological resolution, based on Ct values, following remdesivir treatment in B cell deficiency have been reported.[7] Contrary to these findings we note cell culture demonstrated persisting replication-competent virus. Viral cell cultures appear to be of additional value in understanding infection dynamics: our findings caution

against the use of Ct values alone in inferring virological resolution. Ongoing culture/PCR positivity was managed negative pressure room isolation and adherence to infection prevention and control procedures until discharge from hospital. Phylogenetic analysis of WGS of SARS-COV-2 isolated from hospitalised patients and staff over the same period did not demonstrate temporally associated onward transmission.

While there was no acceleration in the rate of SNPs from an estimated baseline (1-2/month) during persistent infection, there was a relative increase (5 SNPs) following remdesivir treatment, suggesting an increased rate of SARS-CoV-2 mutation,[15] although no significant changes were noted in the remdesivir-binding portion (**Figure 2**). This is in keeping with observation of viral evolution during treatment of chronic SARS-CoV-2 reported elsewhere.[16]

Failure to detect SARS-CoV-2 specific antibodies is unsurprising. A T-cell response however was noted, despite treatment throughout with low-dose tacrolimus. The detection of adequate SARS-CoV-2 specific T-cell responses adds to growing recognition that T-cell mediated immunity can lead to eventual viral resolution, even in the absence of antibodies,[17] albeit at an extended pace.

Our report is limited by discussion of a single case. Additional examples may help to define the potential role for viral cell culture further. One such example may be in supporting infection prevention control decisions. Potential for false negative SARS-CoV-2 PCR swabs are recognised and it may therefore be prudent to acquire serial negative swabs prior to increase confidence prior to chemotherapy based on our findings.

Conclusions

Immunomodulation may have contributed to replication of residual competent virus in this case, while immune stimulation with G-CSF, and subsequent neutrophil reconstitution, may have contributed to relapsing, symptomatic pneumonitis. Viral cell culture alongside WGS has the potential to support further SARS-CoV-2 treatment decisions in such situations.

Immunodeficient patients with persistent SARS-CoV-2 infection several months after initial infection may harbour potential for onward transmission of replication-competent virus. The risk of recurrent pneumonitis should be considered when planning immunomodulatory treatment.

References:

1. Shah V, Ko Ko T, Zuckerman M, Vidler J, Sharif S, Mehra V *et al.* Poor outcome and prolonged persistence of SARS-CoV-2 RNA in COVID-19 patients with haematological malignancies; King's College Hospital experience. B J Haematol. 2020. doi: 10.1111/bjh.16935 [Epub ahead of print].
2. Bhogal T, Khan UT, Lee R, Stockdale A, Hesford C, Potti-Dhananjaya V *et al*. Haematological malignancy and nosocomial transmission are associated with an increased risk of death from COVID-19: results of a multi-center UK cohort. Leuk Lymphoma. 2021; 1-10. doi: 10.1080/10428194.2021.1876865. Online ahead of print.
3. Piralla A, Ricchi M, Grazia Cusi M, Prati P, Vicari N, Scarsi G *et al.* Residual SARS-CoV-2 RNA in nasal swabs of convalescent COVID-19 patients: Is prolonged quarantine always justified? 2021; Int J Inf Dis. 102:299-302. doi: 10.1016/j.ijid.2020.10.072.
4. Graham MS, Sudre CH, May A, Antonelli M, Murray B, Varsavsky T *et al*. Changes in symptomatology, reinfection, and transmissibility associated with the SARS-CoV-2 variant B.1.1.7: an ecological study. Lancet Public Health. 2021; 6(5):e335-e345. doi: 10.1016/S2468-2667(21)00055-4.
5. Zirui Tay M, Meng Poh C, Renia L, Macary PA, Ng LFP. The trinity of COVID-19: immunity, inflammation and intervention. Nat Rev Immunol. 2020; 20(6):363-374. doi: 10.1038/s41577-020-0311-8.
6. Wang X, He Z, Zhao X. Immunoregulatory therapy strategies that target cytokine storms in patients with COVID-19 (Review). Exp Ther Med. 2021; 21(4):319. doi: 10.3892/etm.2021.9750.
7. Buckland MS, Galloway JB, Fhogartaigh CN, Meredith L, Provine NM, Bloor S *et al*. Treatment of COVID-19 with remdesivir in the absence of humoral immunity: a case report. Nature Comms. 2020; 11: 6358.
8. Public Health England. Guidance: Investigation and management of suspected SARS-CoV-2 reinfections: a guide for clinicians and infection specialists. https://www.gov.uk/government/publications/covid-19-investigation-and-management-of-suspected-sars-cov-2-reinfections/investigation-and-management-of-suspected-sars-cov-2-reinfections-a-guide-for-clinicians-and-infection-specialists. Accessed 15 May 21.
9. Prendecki M, Clarke C, Brown J, Cox A, Gleeson S, Guckian M *et al.* Effect of previous SARS-CoV-2 infection on humoral and T-cell responses to single-dose BNT162b2 vaccine. Lancet. 2021 Mar 27;397(10280):1178-1181. doi: 10.1016/S0140-6736(21)00502-X.

10. Prostasio Veras F, Cornejo Pontelli M, Meirelles Silva C, Toller-Kawahisa JE, de Lima M, Carvalho Nascimento D *et al.* SARS-CoV-2–triggered neutrophil extracellular traps mediate COVID-19 pathology. J Exp Med. 2020; 217(12):e20201129. doi: 10.1084/jem.20201129.
11. Schurink B, Roos E, Radonic T, Barbe E, Bouman CSC, de Boer HH *et al.* Viral presence and immunopathology in patients with lethal COVID-19: a prospective autopsy cohort study. Lancet Microbe 2020; 1(7): E290-299. Doi: 10.1016/S2666-5247(20)30144-0.
12. Seddiki N, French M. COVID-19 and HIV-Associated Immune Reconstitution Inflammatory Syndrome: Emergence of Pathogen-Specific Immune Responses Adding Fuel to the Fire. Front Immunol. 2021. 12:649567. doi: 10.3389/fimmu.2021.649567.
13. Malek AE. Time to revisit the use of G-CSF after allogeneic haematopoietic cell transplantation in COVID-19 era? Br J Cancer. 2021; 124:1183. doi: 10.1038/s41416-020-01195-8.
14. Mertens J, Laghrib Y, Kenyon C. A Case of Steroid-Responsive, COVID-19 Immune Reconstitution Inflammatory Syndrome Following the Use of Granulocyte Colony-Stimulating Factor. Open Forum Infect Dis. 2020; 7(8): ofaa326. doi: 10.1093/ofid/ofaa326.
15. Barclay W, Evans C, Darby A, Rambaut A, Pybus O, Robertson D *et al.* NERVTAG report: Is there evidence for genetic change in SARS-CoV-2 and if so, do mutations affect virus phenotype? 2020. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/928724/S0790_NERVTAG_-_Is_there_evidence_for_genetic_change_in_SARS-CoV-2.pdf. Last accessed 05 May 2021.
16. Boshier FAT, Pang J, Penner J, Hughes J, Parker M, Shepherd J *et al.* Remdesivir induced viral RNA and subgenomic RNA suppression, and evolution of viral variants in SARS-CoV-2 infected patients. medRxiv. 2020. Accessed on 14 May 21. doi: 10.1101/2020.11.18.20230599.
17. Clarke CL, Prendecki M, Dhutia A, Gan J, Edwards C, Prout V *et al.* Longevity of SARS-CoV-2 immune responses in hemodialysis patients and protection against reinfection. Kidney Int. 2021. S0085-2538(21)00295-7. doi: 10.1016/j.kint.2021.03.009.

Declarations

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Authors' contributions

SJCP, RW, JY, CP and TB designed the study. CP and MH carried out molecular diagnostics. JT and NK located nasopharyngeal swabs, performed nucleic extraction and managed results. KL and IM performed WGS. AW provided analysis of virus lineages. EG performed viral cell culture. SJCP, RW, LH, NK and MA analysed the data. SJCP and RW drafted the initial manuscript with all authors contributing significantly to revising this for submission. All authors reviewed the results and data analysis and contributed comments. All authors agreed on the final version for submission to the journal.

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Potential conflicts of interests

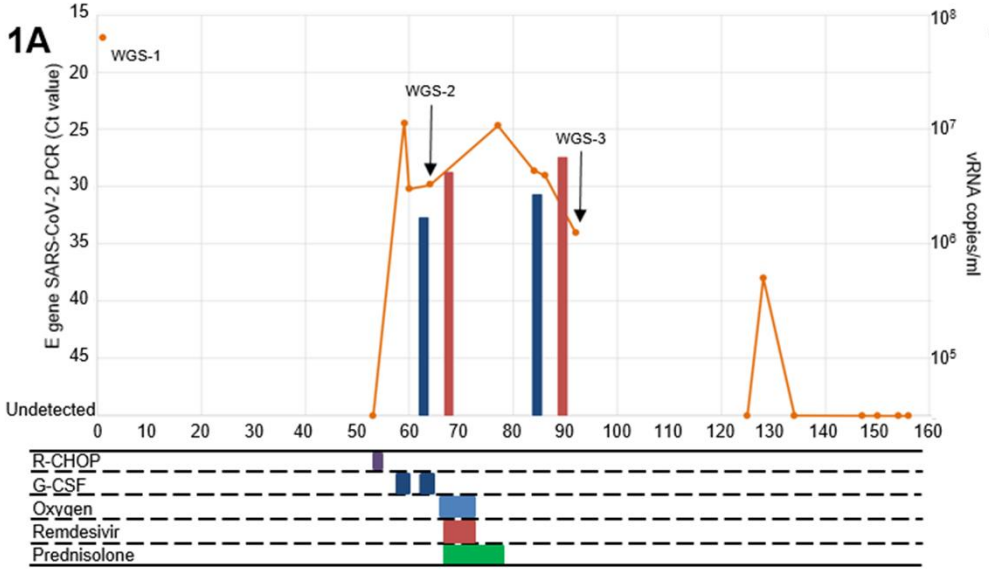
TB has received research funding and speaker fees from Gilead sciences and Pfizer. SJCP has received a research grant from the Scientific Exploration Society supported by the Viscount Gough. PK has received support to use the T-SPOT Discovery SARS-CoV-2 from Oxford Immunotec. All other authors have no conflicts of interest to declare.

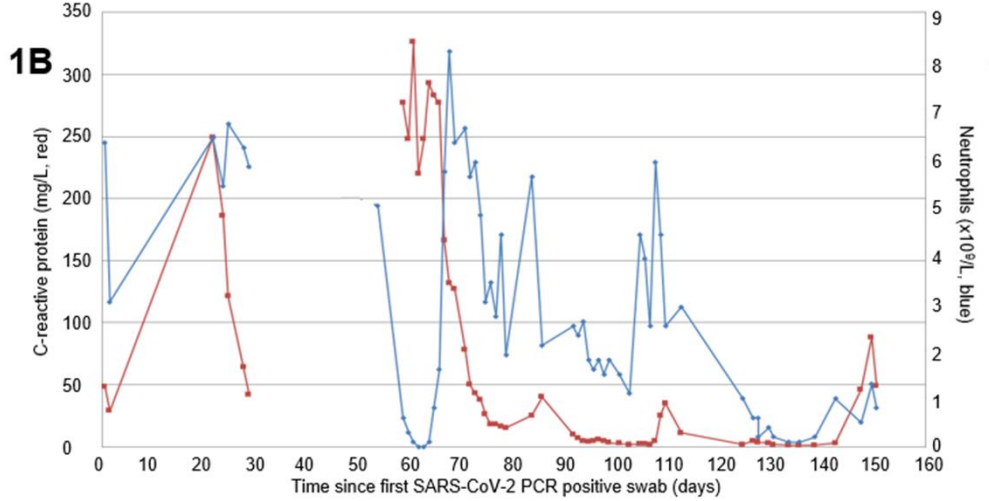
Availability of data and materials

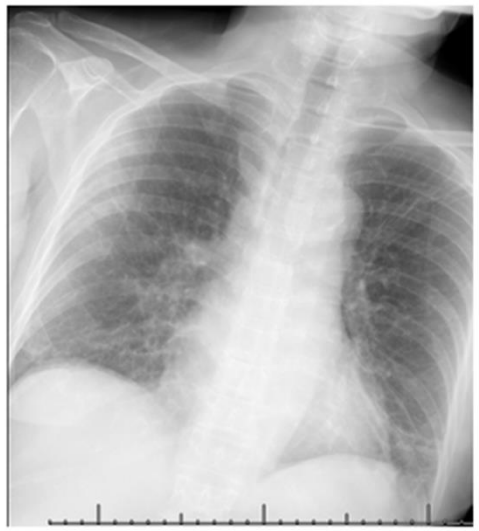
The data analysed during the current study and further details on the assays are available from the corresponding author (SJCP; scott.pallett@nhs.net) on reasonable request, as long as this meets local ethical and research governance criteria.

Ethics

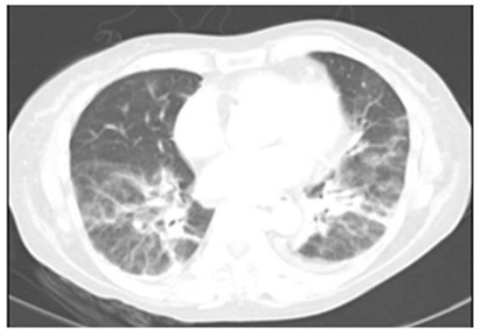
SARS-CoV-2 PCR testing was carried out as part of routine clinical activity. WGS and immunological testing was carried out while investigating for possible SARS-CoV-2 reinfection following consultation with infectious diseases specialist advice and in line with current Public Health England guidance for investigation of possible cases of SARS-CoV-2 reinfection. Viral cell culture was completed in order to investigate possible ongoing infectious potential and guide decision making on in-patient management (in isolation on infectious diseases ward versus under the direct care of the haematology team on the haematology unit and was therefore, as per National Research Ethics Service guidance, was not considered research activity. Informed written consent for case publication was provided by the patient. The case has been reported in line with the CARE guidelines and in compliance with the Declaration of Helsinki principles.


1A
WGS-1
WGS-2
WGS-3
E gene SARS-CoV-2 PCR (Ct value)
vRNA copies/ml
Undetected
R-CHOP
G-CSF
Oxygen
Remdesivir
Prednisolone


1B
C-reactive protein (mg/L, red)
Neutrophils (x10⁹/L, blue)
Time since first SARS-CoV-2 PCR positive swab (days)

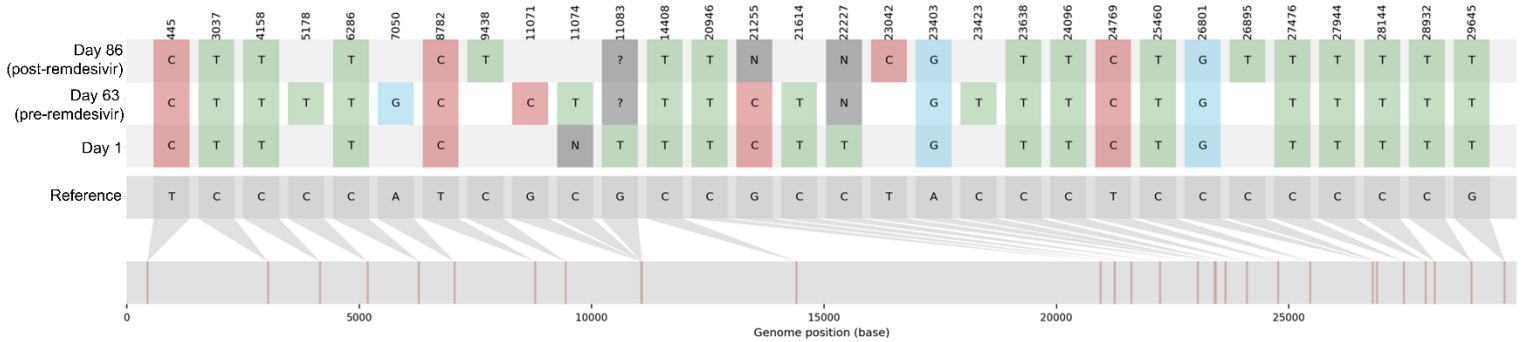

1C

Day 62


1D

Day 66

Figure 1. Assessment of active, *in vitro* SARS-CoV-2 viral cell replication set against clinical infection. *Second positive result on day 21 was provided by the BioFire FilmArray which is unable to provide a Ct value. WGS= Whole genome sequencing.* (**1A**) Change in SARS-CoV-2 PCR results with corresponding Ct values for the E (envelope) gene (orange). Bar graphs show viral RNA copies/ml from an initial NP-swab samples (p0 for each assessment in blue) and after its passage in cell culture (p1 for each sample in red). WGS-*n* highlights those samples that underwent whole genome sequencing (arrows). (**1B**) Timeline of variation in CRP (red) and neutrophil cell count (blue). The central panel marks timing of key interventions, or changes in clinical condition. (**1C**) A chest x-ray conducted on day 62 showing clear lung fields except for few areas of linear atelectasis (**1D**) On day 66 a CT thorax, abdomen, pelvis showed patchy bilateral opacities, with predilection for peripheral and posterobasal distribution, representing evolving COVID-19 pneumonitis.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23042
23403
23423
23638
24096
24769
25460
26801
26895
27476
27944
28144
28932
29645
Day 86 (post-remdesivir)
Day 63 (pre-remdesivir)
Day 1
Reference
0
5000
10000
15000
20000
25000
Genome position (base)

Figure 2. Single nucleotide polymorphisms in the B.1.177.5 SARS-CoV-2 lineage over time pre- and post-challenge with remdesivir. Whole Genome Sequencing was conducted as described in Supplementary information. Post-sequencing analysis was conducted through CIVET version (v) 2.0, utilising several software packages (Python v3.6.13, Matplotlib v3.3.4, Pandas v1.1.0, Tabulate v0.8.9, CSV v1.0, Numpy v1.19.5, Scipy v1.5.3). Baltic COVID-19 Genomics data from 03 May 2021 was used as background data. 3 SNPs were observed between sample 1 (day 1) and sample 2 (day 63) taken pre-remdesivir while a further 5 SNPs were observed between sample 2 (day 63, pre-remdesivir) and sample 3 (day 86, post-remdesivir). WGS was unable to identify 3 nucleotides on the day 63 sample (denoted as ? or N), and unable to identify 2 nucleotides on the day 86 sample.

Supplementary information

(i) Immunological testing

Serum was tested for antibodies to nucleocapsid protein (anti-NP, qualitiative Abbott Architect SARS-CoV-2 IgG 2-step chemiluminescent immunoassay [CMIA]) per manufacturer's instructions. Spike protein antibodies (anti-S) were detected using the quantitative Abbott Architect SARS-CoV-2 IgG Quant II CMIA, with a threshold value for positivity of 7.1 BAU/ml. SARS-CoV-2 specific T-cell responses were detected using the T-SPOT® Discovery SARS-CoV-2 (Oxford Immunotec) as per manufacturer's instructions.[1] In brief, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples with the addition of T-Cell Select™ (Oxford Immunotec) where indicated. 250,000 PBMCs were plated into individual wells.[1] The assay measures immune responses to SARS-CoV-2 structural peptide pools; S1 protein, S2 protein, and positive (phytohemagglutinin) and negative controls. Cells were incubated and interferon-γ secreting T cells detected. Spot forming units (SFU) were detected using an automated plate reader (Autoimmun Diagnostika). Infection-naïve, unvaccinated participants were used to identify a threshold for a positive response using mean +3 standard deviation SFU/10^6 PBMC, as previously described.[9] This resulted in a cut-off for positivity of 40 SFU/10^6 PBMC.[1]

(ii) SARS-CoV-2 RNA PCR and WGS

SARS-CoV-2 RNA PCR was conducted on nasopharyngeal swabs using the Altona or Roche SARS-CoV-2 PCR assay. Cycle threshold (Ct) values were provided for E and S gene targets (Altona) or E and ORF-1a/b gene targets (Roche). Individual PCR melt-curves were analysed to confirm positive/negative results. Oxford nanopore sequencing was carried out using the ARTIC Lo-cost protocol and ARTIC version 3 primers (see below). Comparison of sequenced lineages was conducted to observe SNPs (**Figure 2**).

(iii) Viral replication

Nasopharyngeal-swabs (200µl) were used to inoculate Vero E6 cells (ATCC), grown in Dulbecco's modified Eagle Medium (DMEM) supplemented with heat-inactivated 10% foetal bovine serum (Gibco), 100 U/ml penicillin and streptomycin (Merck) and 2.50 ug/ml

amphotericin B (Merck). Inoculated cells were incubated for 4 days and then subjected to one cycle of freeze-thawing. Lysates (100µL) were processed for RNA extraction (Trizol and Direct-zol RNA kit, Zymo) and qRT-PCR was performed using Fast SYBR Green Master Mix (BioRad) using primers targeting an internal sequence of E.[2] Viral RNA copies were interpolated from a standard curve of *in vitro* transcribed E RNA.

(iv) nCoV-2019 sequencing protocol v3 (LoCost) V.3

Oxford nanopore sequencing of SARS-COV2 was performed essentially as outlined in the Artic Lo-cost protocol using the Artic version 3 primers (https://github.com/artic-network/artic-ncov2019/tree/master/primer_schemes/nCoV-2019/V3) these generate approximately 400 bp amplicons that overlap by around 20 bp.

RT/cDNA preparation

Viral RNA from all clinical samples had a Ct of less than 32 utilising the residual RNA from clinical testing. This was reverse transcribed using LunaScript reverse transcriptase (NEB E3010S) in a final volume of 10ul per reaction with 8ul of Viral RNA. RNA that was stored at -80^{o}C previously frozen, was briefly mixed by vortexing and pulse spin to collect liquid. RNA was maintained on ice where possible.

Sample addition was performed in a class 2 cabinet and was both cleaned and UV irradiated prior to and following procedures A master mix was used for batch processing of samples in a PCR strip-tubes/plate with appropriate controls.

The reaction was incubated for 25 °C for 02 min, followed by 55 °C for 10 min and the enzyme heat denatured at 95 °C for 1 min and was then held at 4 °C until required.

IDT Artic nCOV-V3 primer pools were diluted in molecular grade water, to generate two pools of working 10 µM primer stocks and were used at a final concentration of 15 nM per primer. Pools 1 and 2 have 110 primers and 108 primers respectively.

Multiplex PCR

Two PCR reactions were set up per sample in a class 2 cabinet that was cleaned with decontamination wipes and UV sterilised before and after use. Reagents were gently mixed by pipetting and pulse spun.

Q5 Hot Start High-Fidelity 2X Master Mix (NEB M0494) was used and made up to a final reaction volume of 25µl nuclease free water. Reactions contained 4 µl respectively of either working dilution of the primer pool and 2.5 µl cDNA to each of the PCR reactions. PCR was performed using heat activation of the enzyme at 98°C for 30 sec, 35 cycles 98°C for 15 sec followed by 63°C for 5 mins. On completion the reaction was held at 4°C until required.

Pooling of PCR and quantification

Paired PCR reactions were pooled to make 50 µl per sample (for larger sample numbers this was performed in a PCR plate with the appropriate magnetic base) and cleaned using equal volume AMpure beads (Beckton Dickinson).

In short the mixture was incubated for 2 min at RT applied to a magnet mag followed by removal of the supernatant. Beads were 2 x washed on the magnet with freshly diluted 80% ethanol. The excess ethanol was removed and allowed to briefly air dry for 30 secs. Beads were resuspended in 30 µl nuclease free water by gently pipetting after incubation at RT for 2 mins samples were returned to the magnet and the supernatant retained.

Sample concentration was determined by either Qubit or QuantIT Hs DNA assay (Life Technologies).

Barcode the amplicon pools using the one-pot native barcoding approach.

End Repair and tailing of amplicons was performed using a two step reaction. Initially 6.6 µl of the pooled and cleaned PCR reaction was combined with Ultra II end prep reaction buffer (1.2 µl) and 0.5µl Ultra II End Prep Enzyme Mix (E7546) in 10 µl reaction volume and incubated at 20°C for 15 mins. The enzyme was in activated by incubation at 65 °C for 15 mins and then cooled on ice.

ONT native barcode expansion packs (EXP-NBD104 (1-12), EXP-NBD114 (13-24) or EXP-NBD196) were used to index samples. This was performed by the addition of 1-4 µl of the end repair reaction (according to post PCR clean-up concentration). This was performed in a final reaction volume of 10 µl using 1.25 µl of barcoded adaptor and 2x Blunt/TA Ligase Master Mix. Ligation was performed at 20°C for 20 mins followed by inactivation at 65°C for 10 mins. The reaction was then cooled on ice until required.

12-24 barcoded samples were pooled together and 0.4x v of AMpure beads were added and mixed gently. DNA was Incubated for 5 mins at room temperature. The mix was placed on a magnetic rack and the supernatant discarded. The beads were washed 2x by adding 200 µl SFB (ONT) was added and the beads resuspended completely by pipette mixing. The tubes were replaced on the magnet and the supernatant discarded followed by a single wash with 200 µl freshly made 70 % volume ethanol without resuspension on magnet discarding the ethanol.

Residual ethanol was removed and the pellet allowed to dry for 1 min . The pellet was resuspended in 30 µl 10 mM Tris pH 8.0, followed by incubation for 2 mins at RT and then replaced on magnet and the eluate retained.

The DNA concentration was determined using the QuantIT or Qubit Fluorometery.

AMII adapter ligation was performed with a 30 µL or a maximum 200ng input of the Barcoded amplicon pool, 5 µL Adapter Mix (ONT), using the Quick ligation module 5x buffer and 5 µL Quick T4 DNA ligase (NEB E6056) in a 50 µL reaction volume. The reaction was Incubated at room temperature for 20 min.

On completion purify the DNA by Adding 1:1 volume Ampure beads and mixing. Incubate for 5 min at room temperature. Place on magnetic rack after 2min remove and discard the supernatant. Wash the pellet 2x with 250 µl SFB (ONT) resuspending beads completely by pipette mixing. Residual SFB was removed and beads resuspend in 15 µl EB (ONT) followed by incubating RT for 2 min. Prior to replacing on the magnetic rack. Retaining the eluate.

The library concentration was again determined using the Qubit HS DNA assay.

The flowcell was primed according to the makers instruction and the library diluted for sequencing. Approximately 90 ng of library was made up to 12ul with EB (ONT) or 12 µL of the library was prepared as per the standard instructions for library dilution prior to loading.

References:

1. Prendecki M, Clarke C, Brown J, Cox A, Gleeson S, Guckian M *et al.* Effect of previous SARS-CoV-2 infection on humoral and T-cell responses to single-dose BNT162b2 vaccine. Lancet. 2021 Mar 27;397(10280):1178-1181. doi: 10.1016/S0140-6736(21)00502-X.
2. Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DKW *et al.* Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 2020; 25(3): 2000045. doi: 10.2807/1560-7917.ES.2020.25.3.2000045.