

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

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 *versus* 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 pneumonia, 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 pneumonia 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, in-house whole-genome sequencing (WGS) and immunological testing using both B-cell and T-cell assays (Data S1).^{8,9} Additionally, in-house viral cell culture was conducted to assess for replication-competent virus (Data S1).

Case

A 65-year-old lady who underwent a 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 6 g/l for which she was managed expectantly.

In November 2020, she presented acutely with abdominal pain. Computed tomography (CT) imaging demonstrated a bulky retroperitoneal soft tissue mass at the porta hepatis, associated with 4 kg of weight loss. A bone marrow aspirate demonstrated infiltration with a CD19-positive, CD5-negative B-cell NHL. Gastroscopy showed abnormal appearance of gastric folds and gastric biopsy confirmed CD5⁺ 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 ($0.0 \times 10^9/l$) and fever ($38^\circ C$) subsequently developed (day 58; Fig 1A,B). Lymphocyte count was $0.0 \times 10^9/l$, remaining $<1.0 \times 10^9/l$ 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, Fig 2). Six doses of granulocyte colony-stimulating factor (G-CSF) were administered on days 58–60, 62–64 (neutrophil nadir 0.0, peak $6.6 \times 10^9/l$). Tacrolimus levels remained within range. New bilateral pulmonary infiltrates on chest imaging (Fig 1), a C-reactive protein (CRP) level of 326 mg/l and a rising neutrophil count followed (day 66), alongside new oxygen requirements. Investigation for possible co-infection included urinary, sputum, serial blood cultures as well as carrying out PCR on ethylenediamine tetra-acetic acid (EDTA) blood for cytomegalovirus (CMV) and adenovirus. Atypical urinary antigens and a syndromic respiratory PCR panel (Biofire Respiratory 2-1, BioFire Diagnostics [bioMérieux], Salt Lake City, UT, USA) targeting 22 virus and bacteria targets were also conducted. All results were negative. Increased prednisolone (40 mg daily, tapering 10 mg daily every 5 days) and remdesivir (200 mg stat, 100 mg daily for 5 days) were commenced (day 67): fever, dyspnoea and oxygen requirement resolved, and CRP declined to 22 mg/l (Fig 1B).

PCR tests remained positive on days 76 [decreased cycle threshold number (Ct) value], 83, 86 and 91, becoming negative from day 134 onwards (Fig 1A). Viral cell culture performed on day 64 and 86 (before and after remdesivir, with

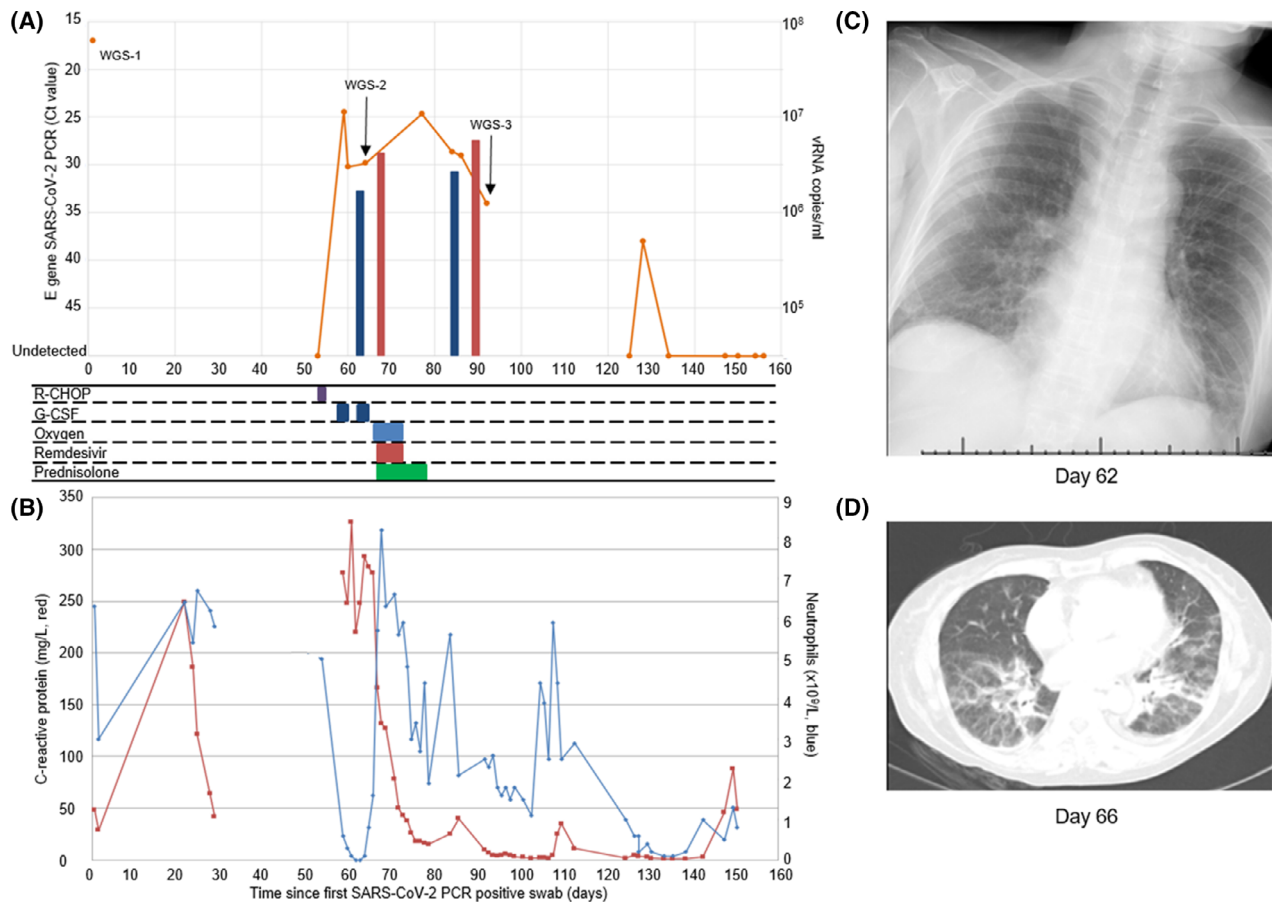


Fig 1. Assessment of active, *in vitro* SARS-CoV-2 viral cell replication set against clinical infection. Second positive result on day 21 was provided by the BioFire FilmArray which is unable to provide a threshold cycle number (Ct) value. WGS = Whole genome sequencing. (A) Change in SARS-CoV-2 PCR results with corresponding Ct values for the E (envelope) gene (orange). Bar graphs show viral RNA copies/ml from 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). (B) Timeline of variation in C-reactive protein (CRP, red) and neutrophil cell count (blue). The central panel marks timing of key interventions, or changes in clinical condition. (C) A chest X-ray conducted on day 62 showing clear lung fields except for few areas of linear atelectasis (D) On day 66, a computed tomography (CT) thorax, abdomen, pelvis scan showed patchy bilateral opacities, with predilection for peripheral and posterobasal distribution, representing evolving COVID-19 pneumonitis. [Colour figure can be viewed at wileyonlinelibrary.com]

both swabs also PCR-positive) demonstrated replication-competent virus [approximately 400-fold increase of detectable RNA over inoculum (Fig 1)]. WGS of viral RNA showed ongoing infection with the same B.1.177.5 lineage throughout (Fig 2), acquiring three single nucleotide polymorphisms (SNPs) between days 1 and 63 and a further five SNPs between days 64 and 86 (after remdesivir).

SARS-CoV-2-specific anti-nucleocapsid and anti-spike (anti-S) antibodies were negative, whereas enzyme-linked immunosorbent 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 the pro-inflammatory cytokines interleukin-1, tumour necrosis

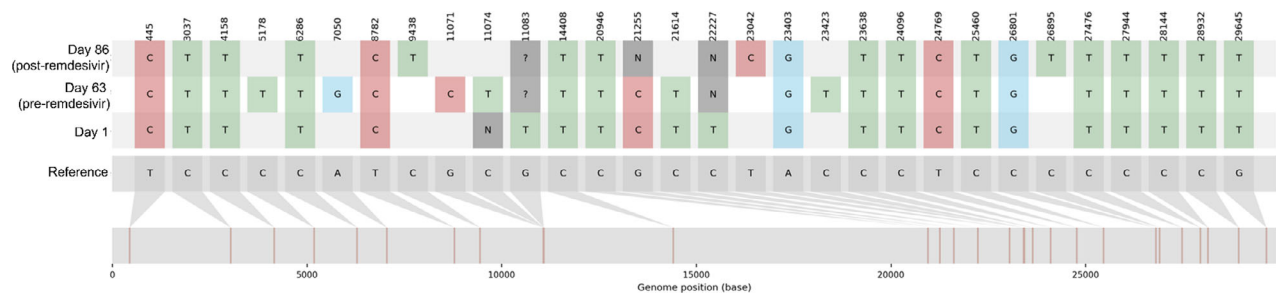


Fig 2. Single nucleotide polymorphisms (SNPs) in the B.1.177.5 SARS-CoV-2 lineage over time pre- and post-challenge with remdesivir. Whole-genome sequencing (WGS) was conducted as described in the Supplementary information. Post-sequencing analysis was conducted through CIVET version (v) 2.0, utilising 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 were used as background data. Three SNPs were observed between sample 1 (day 1) and sample 2 (day 63) taken pre-remdesivir while a further five SNPs were observed between sample 2 (day 63, pre-remdesivir) and sample 3 (day 86, post-remdesivir). WGS was unable to identify three nucleotides on the day 63 sample (denoted as? or N), and unable to identify two nucleotides on the day 86 sample. [Colour figure can be viewed at wileyonlinelibrary.com]

factor (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; Fig 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 (Fig 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 a 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 by 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 (five 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 (Fig 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 was noted, however, despite treatment throughout with low-dose tacrolimus. The detection of adequate SARS-CoV-2-specific T-cell responses adds to the 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. The potential for false negative SARS-CoV-2 PCR swabs is recognised and it may therefore be prudent to acquire serial negative swabs 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.

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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, not considered research activity. Informed written consent for case publication was provided by the patient. The case has been reported in line with the case report (CARE) guidelines and in compliance with the Declaration of Helsinki principles.

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Author 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.

Conflicts of interest

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.

Data availability statement

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.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Detailed methods are described for (i) immunological testing, (ii) SARS-CoV-2 RNA PCR testing, (iii) in-

house viral cell culture and (iv) in-house SARS-CoV-2 whole genome sequencing protocol.

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