



RESEARCH ARTICLE

REVISED SARS-CoV-2 RNA detected in blood products from patients with COVID-19 is not associated with infectious virus [version 2; peer review: 2 approved]

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Abstract

Background: Laboratory diagnosis of SARS-CoV-2 infection (the cause of COVID-19) uses PCR to detect viral RNA (vRNA) in respiratory samples. SARS-CoV-2 RNA has also been detected in other sample types, but there is limited understanding of the clinical or laboratory significance of its detection in blood.

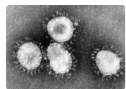
Methods: We undertook a systematic literature review to assimilate the evidence for the frequency of vRNA in blood, and to identify associated clinical characteristics. We performed RT-PCR in serum samples from a UK clinical cohort of acute and convalescent COVID-19 cases (n=212), together with convalescent plasma samples collected by NHS Blood and Transplant (NHSBT) (n=462 additional samples). To determine whether PCR-positive blood samples could pose an infection risk, we attempted virus isolation from a subset of RNA-positive samples.

Results: We identified 28 relevant studies, reporting SARS-CoV-2 RNA in 0-76% of blood samples; pooled estimate 10% (95%CI 5-18%). Among serum samples from our clinical cohort, 27/212 (12.7%) had SARS-CoV-2 RNA detected by RT-PCR. RNA detection occurred in samples up to day 20 post symptom onset, and was associated with more severe disease (multivariable odds ratio 7.5). Across all samples collected ≥ 28 days post symptom onset, 0/494 (0%, 95%CI 0-0.7%) had vRNA detected. Among our PCR-positive samples, cycle threshold (ct) values were high (range 33.5-44.8), suggesting low vRNA copy numbers. PCR-positive sera inoculated into cell culture did not produce any cytopathic effect or yield an increase in detectable SARS-CoV-2 RNA. There was a relationship between RT-PCR negativity and the presence of total SARS-CoV-2 antibody (p=0.02).

Conclusions: vRNA was detectable at low viral loads in a minority of serum samples collected in acute infection, but was not associated with infectious SARS-CoV-2 (within the limitations of the assays used). This work helps to inform biosafety precautions for handling blood products from patients with current or previous COVID-19.

Keywords

COVID-19, SARS-CoV-2, viral load, viraemia, RNA, blood, biomarker, laboratory safety



This article is included in the [Coronavirus \(COVID-19\)](#) collection.

Open Peer Review

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Any reports and responses or comments on the article can be found at the end of the article.

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Competing interests: DWE has received personal fees from Gilead, outside the submitted work.

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REVISED Amendments from Version 1

The amended version of this article includes new data reporting total antibody titres measured using the Siemens Attelica immunoassay on a subset of our SARS-CoV-2-positive samples. We show a relationship between antibody-negative status and the presence of RNA-aemia. However, absolute antibody titres were not statistically different in the samples that were RT-PCR positive vs negative, and the lack of cytopathic effect *in vitro* was not dependent on the presence of antibody. The additional data are supported by small changes in the abstract, methods, results and discussion.

Any further responses from the reviewers can be found at the end of the article

Background

Since January 2020, the SARS-CoV-2 virus has caused a global pandemic of COVID-19, challenging hospitals and laboratory services worldwide¹. Diagnosis of infection has largely been based on RT-PCR amplification of viral nucleic acid from the upper respiratory tract (nose/throat) swabs². However, detection of viral RNA (vRNA) has also been reported in blood, serum and plasma from clinical small case series (e.g. 3,4). The frequency and quantification of SARS-CoV-2 RNA in blood fractions, and the significance of blood as a transmission route remains unknown.

Understanding the clinical contexts within which SARS-CoV-2 RNA can be detected in blood is important to determine the extent to which PCR-positive blood, plasma or serum could have impact as a clinically useful biomarker of disease severity or prognosis. Furthermore, there is an urgent need to consider whether the detection of viral RNA in blood samples reflects the presence of infectious virus, as this has important safety implications for clinicians and laboratory personnel engaged in both routine laboratory testing, as well as SARS-CoV-2-specific pipelines such as serology^{5,6}.

Different organisations have made varying recommendations for the laboratory handling of samples from patients with suspected or confirmed SARS-CoV-2, but these have had to be developed quickly in the face of little experience or data, and rely on the presence of viral RNA in samples as an imperfect surrogate for live virus. Laboratory protocols seeking to reduce the bioburden of SARS-CoV-2 in clinical samples suggest either chemical inactivation (e.g. with sodium-dodecyl-sulfate, Triton-X100, and/or guanidinium thiocyanate-lysis buffers), alone or in combination with heating protocols that vary from 30°C up to as high as 92°C for 15 minutes⁷. These approaches add processing time, may require additional laboratory reagents, and are also potentially associated with a loss of sensitivity in any downstream analysis, particularly pertinent for serological assays. Previous reports suggest that heat inactivation may be particularly detrimental to the sensitivity of antibody detection⁸.

An alternative to chemical or heat inactivation is to undertake all sample handling in a biosafety (containment) level 3 (BSL3) facility, but this is expensive, requires specialist staff training, substantially reduces the number of samples that can be processed, and is completely inaccessible in many settings.

There is a lack of consensus about appropriate biosafety precautions, and escalation to BSL3 may be based on concerns about risks associated with viraemic samples even when the risk of aerosol generation is low, and there are no data to suggest a risk of blood-borne transmission to laboratory staff.

Here we assimilate the peer-reviewed literature describing the presence of SARS CoV-2 RNA in human blood, with the aim of providing a pooled dataset to provide improved insights into the causes and correlates of RNA-aemia. We then present our own investigation of the frequency and determinants of vRNA detection in blood using 424 samples collected from acutely infected and convalescent patients infected with SARS-CoV-2. We attempted *in vitro* isolation of the virus from viraemic samples in order to determine whether RNA detection is a marker of infectious virus. Together, these data may help to determine the significance of viral RNA in blood, and can contribute to the development of consistent and evidence-based laboratory protocols.

Methods**Terminology and definitions**

- **Blood:** we have used the term blood to refer to whole blood, serum or plasma when there is not a clear distinction in existing pre-published data, although we recognise that there may be differences in the sensitivity of viral detection between whole blood and blood fractions.
- **Serum:** in the work undertaken here, we refer specifically to serum, as this blood fraction was consistently used across our experiments.
- **RNA-aemia:** we have used this term to describe the presence of viral RNA, above the technical limits of detection of RT-PCR assays, in blood, serum or plasma. The alternative term, ‘viraemia’, suggests the presence of whole virus in blood. Since we have not demonstrated the presence of replication-competent (infectious) SARS-CoV-2 in the blood compartment, we have elected to use the more conservative description of RNA-aemia (which may or not indicate viraemia).

Systematic literature review

We searched PubMed, Web of Science, MedRxiv and Google between 7th-11th May 2020, using the search terms (“SARS-CoV-2” OR “COVID” OR “2019-nCoV” OR “COVID-19” OR “2019 NCOV” or “SARS COV 2” or “2019NCOV” or “2019-nCoV” or “2019 novel coronavirus”) AND (“qPCR” OR “RT-PCR” OR “PCR” OR “VIRAL LOAD” OR “RNAaemia” OR “RNAemia” OR “viraemia” OR “viremia” OR “RNA-aemia” OR “RNA-emia”) AND (“BLOOD” OR “PLASMA” OR “SERUM”). We excluded animal studies. We did not make exclusions on the basis of language, but two papers not in English were ruled out because they did not contain details of vRNA detection that we required. Each study was reviewed by at least two independent reviewers. A PRISMA flow chart is presented, showing identification of 28 relevant studies (Figure 1; Extended Data Table 1⁹). We collected information on the prevalence of vRNA detection in blood, serum or plasma, noting whether this attribute was correlated with clinical or laboratory phenotypes of disease, and recording cycle threshold (Ct) values when these

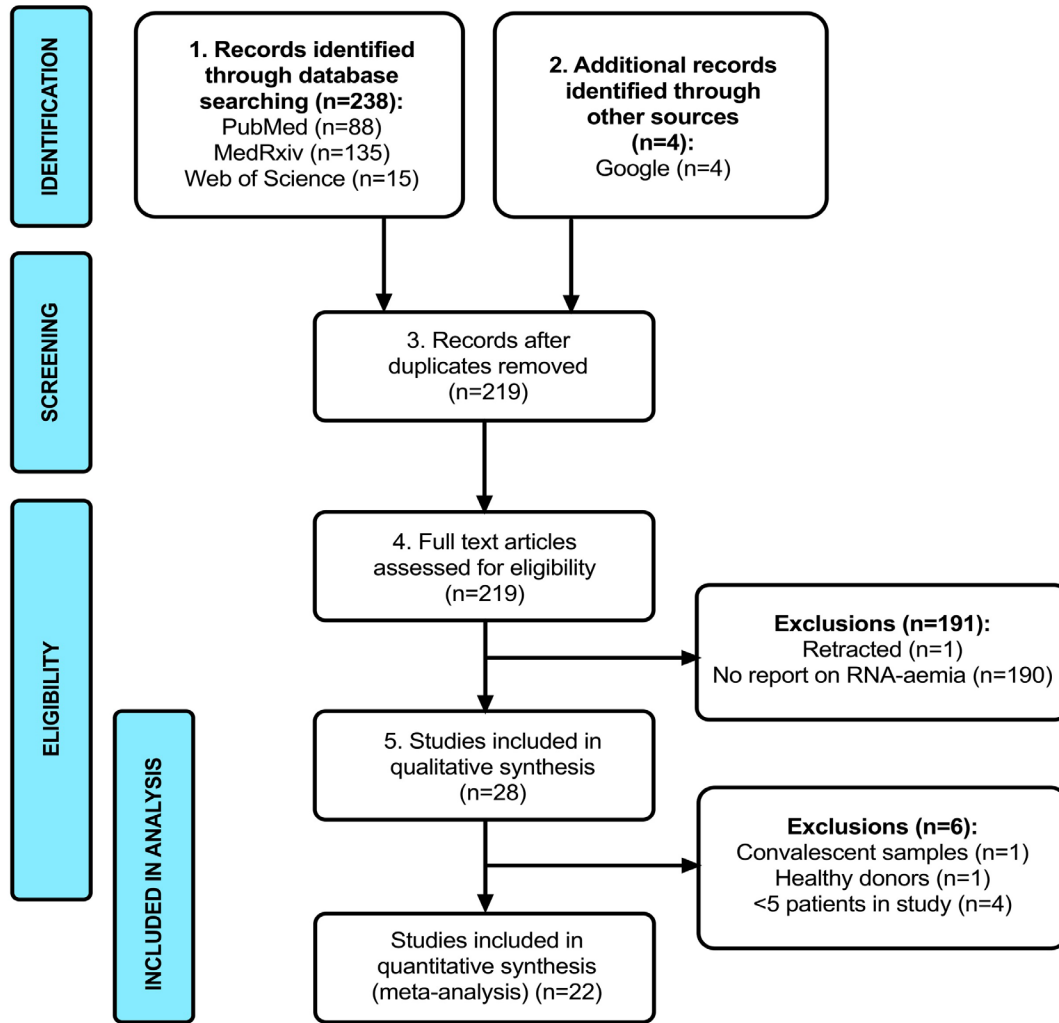


Figure 1. PRISMA flow diagram showing number of abstracts identified through a systematic literature review, rejections (with reasons), and final number of studies included in the analysis.

were reported. Data were collated in Microsoft Excel v16.31. To allow appraisal of quality and identification of bias, we recorded the number of participants in each study, the location and nature of the study cohort, and (where available) the severity of illness and the timing of sample collection relative to symptoms or PCR- diagnosis. To reduce bias in the meta-analysis, we removed one study each of uninfected (healthy) donors and convalescent individuals, and four studies with <5 participants, taking the final number of studies analysed to 22 (Figure 2).

Cohorts and sample selection

The origin of serum samples, together with supporting meta-data, are available in Underlying Data File 1⁹. We collected 212 serum samples through the microbiology department at Oxford University Hospitals NHS Foundation Trust (OUH NHSFT), comprising adults with SARS-CoV-2 infection confirmed by a clinical diagnostic microbiology laboratory using RT-PCR on a

respiratory swab. These were derived from three groups as follows:

- (i) **Hospital in-patients**, n=139 samples from 94 participants; these were collected from individuals admitted to OUH NHSFT, a tertiary referral centre in the South East of England, for treatment of COVID-19. Samples were collected between 1–5 days following admission to hospital or intensive care (whichever came later), a median of 8 days following symptom onset (range 1-37 days).
- (ii) **Convalescent healthcare workers**, n=41 samples from 41 participants; these were collected from healthcare workers from OUH NHSFT, following a period of ≥ 7 days absence from work following a diagnosis of COVID-19, a median of 12 days following symptom onset (range 7–17 days).
- (iii) **Convalescent patients**, n=32 samples from 32 participants; these were collected from patients presenting

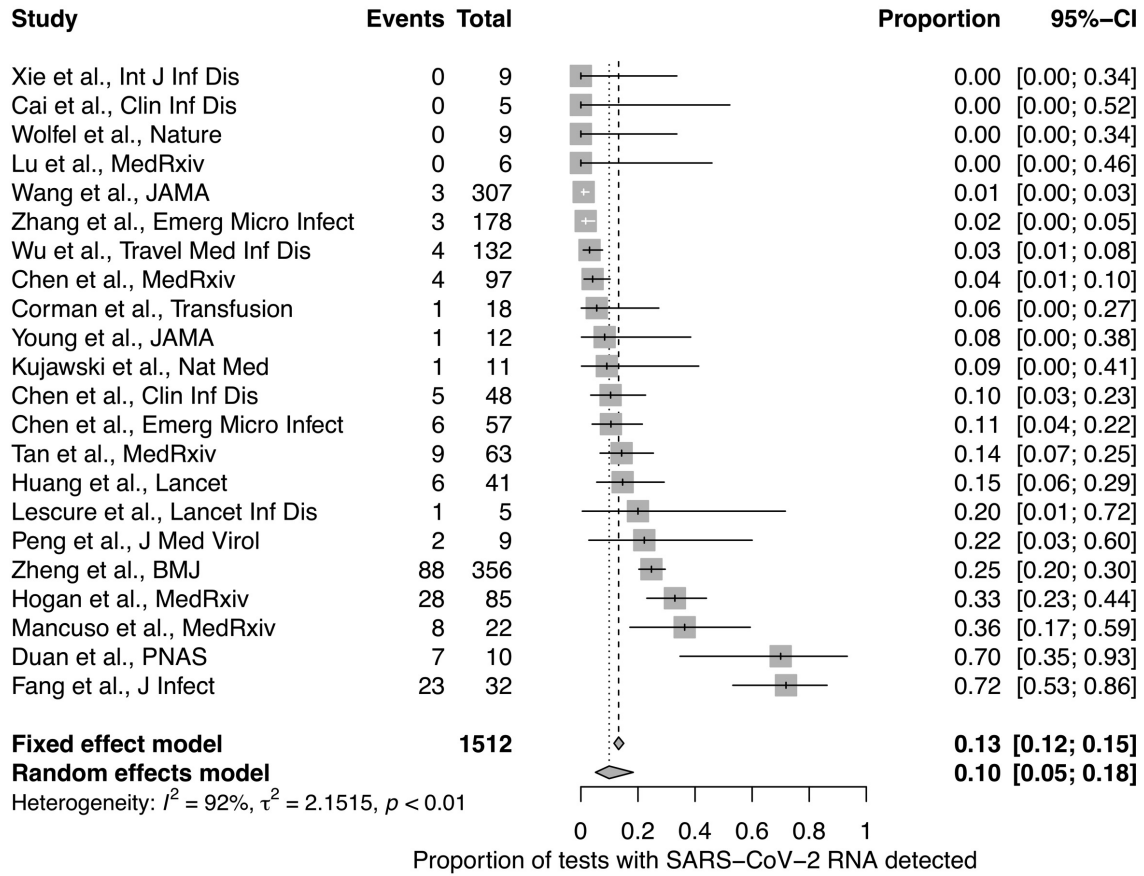


Figure 2. Prevalence of SARS-CoV-2 RNA in serum / plasma / whole blood samples from a systematic literature review. Point prevalence indicated for each study with confidence intervals showing citation and number of samples represented (Table 1).

to OUH NHS FT followed up in the community, a median of 42 days following onset of COVID-19 symptoms (range 31–62 days).

Additional samples were collected through NHS Blood and Transplant (NHSBT), as follows:

- (vi) **Convalescent plasma donors**, n=142 samples from 142 volunteer plasma donors, ≥ 28 days from recovery of symptoms. Retrospective confirmation of COVID-19 infection was based on a EuroImmun IgG antibody titre (threshold ratio ≥ 1.1 , based on the manufacturer’s instructions, Underlying data table 1).
- (v) **Healthy pre-pandemic controls**, n=5 samples from 5 independent healthy volunteer donors, collected prior to December 2019.

In groups (i)–(iii) >1 sample was obtained from 45 individuals, so our clinical dataset overall represents 167 unique individuals

with COVID-19. Among these 167 individuals, we classified severity of illness as asymptomatic, mild, severe, or critical based on standard WHO criteria¹⁰. All serum samples were frozen in 0.5ml aliquots at -20°C.

RT-PCR on serum samples

Following nucleic acid extraction, we used reverse transcription (RT)-PCR to amplify SARS-CoV targets from serum samples. PCR primer sequences are available in a supporting on-line file set⁹. Due to different pathways for patient recruitment and sample processing, PCR protocols varied by cohort, as follows:

- Samples from acute hospital admissions and convalescent health care workers were processed by the OUH NHSFT clinical microbiology laboratory (UKAS accredited to ISO 15189:2012), using a Quiagen Symphony Rotorgene protocol with an RNA-dependent RNA polymerase (RdRP) gene target, validated by Public Health England (PHE) for use on respiratory samples^{11,12}.

Table 1. Frequency of SARS-CoV-2 RNA in human blood and blood products based on a systematic literature review. Full metadata are presented in Extended Data File 1, available online⁹.

Citation	Setting	Frequency and characteristics of SARS-CoV-2 RNA
ACUTE COVID-19 INFECTION		
Wang <i>et al.</i> , <i>JAMA</i> ⁴	n=205 patients with COVID-19; Hubei and Shandong provinces and Beijing, China	<ul style="list-style-type: none"> Blood: 3/307 samples RNA positive collected from 205 patients (0.98%); mean Ct 34-35 No difference in Ct values between blood, stool, and respiratory samples
Zhang <i>et al.</i> , <i>Emerging Microbes & Infections</i> ¹³	n=178; Wuhan pulmonary hospital, China	<ul style="list-style-type: none"> Whole blood: 6/178 (3.4%) PCR positive; Ct 30-32 Serum: 3/178 (1.7%) PCR positive; Ct 24-33 None of the patients with viral RNA detected in blood had positive respiratory swabs
Lescure <i>et al.</i> , <i>Lancet Inf. Dis.</i> ¹⁴	n= 5, hospital patients, France	<ul style="list-style-type: none"> Plasma: 1/5 (20%) PCR positive; Ct >35 Latest positive 12 days after symptom onset. The patient with vRNA-aemia was the most severely ill.
Duan <i>et al.</i> , <i>PNAS</i> ¹⁵	n= 10, severe COVID19 patients, Wuhan, China	<ul style="list-style-type: none"> Serum: 7/10 (70%) PCR positive; Ct 34-38
Chen <i>et al.</i> , <i>CID</i> ¹⁶	n=48, General Hospital of Central Theater Command, PLA, Wuhan, China	<ul style="list-style-type: none"> Serum: 5/48 (10%) PCR positive RNAaemia only in the critically ill group (but 12 critically ill patients had no RNA-aemia) RNA-aemia associated with elevated IL-6
Chen <i>et al.</i> , <i>Emerg Microbes Infect.</i> ¹⁷	n=57, Guangzhou Eighth People's Hospital, China	<ul style="list-style-type: none"> Serum: 6/57 (11%) PCR positive; Ct 32-41 RNA-aemia associated with severe symptoms
Fang <i>et al.</i> , <i>J. Infect.</i> ¹⁸	n=32, Central Hospital of Xiangtan, China	<ul style="list-style-type: none"> Blood: 7/8 (88%) PCR positive in ICU patients and 16/24 (67%) in non-ICU patients.
Han <i>et al.</i> , <i>CID</i> ¹⁹	n=2, Seoul Metropolitan Government-Seoul National University, Korea	<ul style="list-style-type: none"> Mother and 27 day old infant Plasma: RNA detected in infant up to day 10, mother's plasma negative
Huang <i>et al.</i> , <i>Lancet</i> ²⁰	n=41, hospitalised patients, Jin Yin-tan Hospital, Wuhan, China	<ul style="list-style-type: none"> Plasma: 6/41 (15%) PCR positive No difference in ICU admissions between patients with and without RNA-aemia.
Yu <i>et al.</i> , <i>CID</i> ²¹	n=4, Beijing Ditan Hospital, Capital Medical University, Beijing, China	<ul style="list-style-type: none"> Blood: 0/4 (0%) PCR positive
Young <i>et al.</i> , <i>JAMA</i> ²²	n= 18, hospitalized patients, Singapore	<ul style="list-style-type: none"> Blood: 1/12 (8%) PCR positive
Xie <i>et al.</i> , <i>Int J Inf Dis.</i> ²³	n=9, Sichuan Provincial People's Hospital and Sichuan Mianyang 404 Hospital, Chengdu, China	<ul style="list-style-type: none"> Blood: 0/9 (0%) PCR positive
Wu <i>et al.</i> , <i>Travel Med Inf Dis.</i> ²⁴	n=132, The East Section of Renmin Hospital of Wuhan University, China	<ul style="list-style-type: none"> Blood: 4/132 (3.03%) PCR positive
Cai <i>et al.</i> , <i>CID</i> ²⁵	n=5, Childrens' hospital, Shanghai	<ul style="list-style-type: none"> Serum: 0/5 PCR positive within 2-3 days of symptom onset
Zheng <i>et al.</i> <i>BMJ</i> ³	n= 96 admitted patients Zhejiang province, China	<ul style="list-style-type: none"> Serum: 39/96 (41%) overall (6/22 (27%) in mild cases, and 33/74 (45%) in severe case) No difference in viral load between mild and severe cases Serum had the lowest viral load compared with stool and respiratory samples.
Wolfel <i>et al.</i> , <i>Nature</i> ²⁶	n=9, hospitalised, Munich, Germany	<ul style="list-style-type: none"> Serum: 0/9 (0%) PCR positive
Kujawski <i>et al.</i> , <i>Nature Medicine</i> ²⁷	n=11, hospitalised patients, USA	<ul style="list-style-type: none"> Serum: 1/11 (9%) PCR positive Detection of RNA in serum associated with clinical deterioration

Citation	Setting	Frequency and characteristics of SARS-CoV-2 RNA
Peng L <i>et al.</i> , <i>J Med Virology</i> ²⁸	n=9, hospitalised patients, Sun Yat-sen University, China	• Whole blood: 2/9 PCR positive
Corman VM <i>et al.</i> , <i>Transfusion</i> ²⁹	n=18, range of patients, Germany	• Serum: 1/18 PCR positive, in patient with ARDS needing mechanical ventilation. • SARS-CoV-2 present at 179 copies/ml
Song <i>et al.</i> , <i>MedRxiv</i> ²⁰	n=1, China	• Plasma: 0/1 positive
Lu <i>et al.</i> , <i>MedRxiv</i> ³¹	n=6, hospitalised patients, Jiangsu, China.	• Serum: 0/6 positive
Mancuso <i>et al.</i> , <i>MedRxiv</i> ²²	n=22 (10 severe disease, 12 mild disease), Milan, Italy	• Plasma: 6/10 RNA positive in severe group (60%) and 2/12 (1.6%) in the mild group.
Hogan <i>et al.</i> , <i>MedRxiv</i> ²³	n=85, California, USA	• Plasma: 28/85 detectable RNA • Median Ct value 37.5 (compared with 27.1 for nasopharyngeal aspirate) • Those with RNA-aemia were older and more likely to go to ICU and need mechanical ventilation • All deaths occurred in those with RNA-aemia
Tan <i>et al.</i> , <i>MedRxiv</i> ³⁴	n=67, Chongqing, China	• 9/63 (14%) positive for RNA
Chen <i>et al.</i> , <i>MedRxiv</i> ²⁵	n=97, Zhuhai, China	• Whole blood: 4/97 • All 4 patients with RNA-aemia had the lowest oxygenation
Bouadma <i>et al.</i> , <i>MedRxiv</i> ³⁶	n=1, Paris, France	• Blood: 1/1 RNA detected • Patient developed multi-organ failure and died
CONVALESCENT PATIENTS (>28 days)		
Ling <i>et al.</i> <i>Chinese Med J</i> ³⁷	n=14, convalescent patients	• Serum: 0/14 (0%)
HEALTHY DONORS		
Chang <i>et al.</i> , <i>Emerging Infectious Diseases</i> ³⁸	n= 7425 Healthy blood donors, Wuhan Blood Center, China. Collected Jan-March 2020, peak epidemic.	• Prospective testing of 1,656 platelet donations and 774 whole blood donations: 1/2430 RNA positive (0.04%) • Retrospective testing of whole blood donations: 3/4995 RNA positive (0.1%)

We used the QIAgen OBL complex 200 extraction method, using QIASymphony DSP virus/pathogen mini kit (Qiagen 937036), adding 200µl of serum sample to 430µl QIASymphony complex off-board lysis buffer (Internal Control MS2 RNA, Sigma-Aldrich 10165948001 (0.5µl); Molecular grade water Fisher Scientific 10245203 (1.5µl); RNA carrier, Qiagen 1017647 (9µl); AVE, Qiagen (109µl); PK, Qiagen 19133 (20µl); ATL, Qiagen 157054504 (100µl); ACL, Qiagen 160030311 (190µl)) and eluting into 60µl. Cycling conditions were 55°C for 10 minutes; 94°C for 3 minutes; 45 cycles of 94°C 15 seconds; 58°C for 30 seconds. A report of Ct values, including positive control on each run, was generated by Rotor-Gene Q series software 2.3.1.

- For convalescent OUH NHSFT patients, a nested PCR was undertaken using newly developed PCR primers at the Medawar Building for Pathogen Research, Oxford, targeting the RNA-dependent RNA polymerase (RdRp)

gene of SARS-CoV-2³⁹. For the first round amplification, we generated a 25 µL reaction mix (5 µL RNA extract; 12.5 µL 2X Quantitect Probe RT-PCR Master Mix (Qiagen 204343); 0.5 µL RT mix from the kit; 5 µL 5X 1st-round primer mix (IDT); 2 µL PCR-grade water). PCR conditions: 50°C for 30 minutes; 95°C for 15 minutes; 40 cycles of 95°C for 15 seconds; 55°C for 30 seconds; 68°C for 1 minute; 68°C for 5 minutes. For the second round amplification, we generated a 25 µL reaction mix (1 µL of the first round product; 5 µL 5X GoTaq Green Master Mix (Promega M7841); 0.125 µL 5u/µL GoTaq G2 polymerase (Promega M7841); 2 µL 2.5 mM dNTP mix (Strattech NU-1020S-JEN-200ul); 5 µL 2nd-round 5X primer mix (IDT); 11.875 µL PCR-grade water). PCR conditions: 95°C for 5 minutes, 40 cycles of 95°C for 30 s, 55°C for 30 seconds, 72°C for 1 minute, final extension of 72°C for 5 minutes. The presence of SARS-CoV-2 RNA was confirmed through visualization of the PCR product via UV-Vis agarose gel electrophoresis.

The assay demonstrated a 95% detection rate for 13 RNA copies of SARS-CoV-2 RNA transcript spanning the amplified region³⁹.

- Convalescent samples collected through NHSBT were analysed by Public Health England (Colindale), targeting either RdRp or a conserved region of the open reading frame (ORF1ab) gene of SARS CoV-2, together with detection of an assay internal control to monitor the extraction and RT-PCR processes. Reverse transcription and PCR amplification was performed on an Applied Biosystems 7500 FAST system. Samples were aliquoted into lysis buffer containing an exogenously added internal control (soil-borne cereal mosaic virus (SBCMV) RNA transcripts⁴⁰), prior to purification of nucleic acid. Total nucleic acid was extracted from samples using the Biomérieux NucliSENS easyMAG or eMAG system. Extracted nucleic acid was analysed by either an RT-PCR assay targeting the RDRP gene of SARS -2 CoV as previously described with minor modifications¹² or a conserved region of the open reading frame (ORF1ab) gene. This assay uses the primers and probe sequences made public by CDC China⁴¹, together with detection of SBCMV IC. ORF1ab primers (100µM) were obtained from Metabion, Planegg, Germany, and ORF1ab probe (10µM) from Tib-Molbiol, Berlin, Germany. A 25µL RT-PCR reaction contained: 5µL of RNA; 12.5µL of 2x reaction buffer; 0.4µL MgSO₄ (50mM) provided with the Superscript III one step RT-PCR system with Platinum Taq Polymerase (Cat.11732088; Invitrogen, Darmstadt, Germany); 0.15µL ORF1ab-F; 0.2µL ORF1ab-R; 0.125µL SBCMV-F; 0.5µL SBCMV-R; 0.5µL ORF1ab-P; 0.125µL SBCMV-P; 1µL of reverse transcriptase/Taq mixture from the kit; 4.5µL molecular grade water. Thermal cycling was performed on an Applied Biosystems 7500 FAST system (Applied Biosystems, Thermo Fisher Scientific, Hemel Hempstead, England), as follows: 55°C for 10 minutes, 94°C for 3 minutes, 45 cycles of 94°C for 15s, 58°C for 30 seconds.

High Ct values (>37.0) are often viewed as being non-specific in clinical diagnostic laboratories depending on the clinical situation. However, for research purposes we collected and reported all Ct values.

Viral culture system

For viral culture, we used 20 serum samples, designated VC01-20 (identified in Underlying Data File 1⁹). VC01-16 comprised acute and convalescent samples that were RT-PCR positive, selected at random from our sample bank, representing samples from 12 individual patients (four individuals were represented at two timepoints), collected at 3-20 days following onset of symptoms. VC17-20 were pre-pandemic control samples. One further sample collected from a pre-pandemic NHSBT serum donation was used as media (VC21).

Samples VC01-20 were provided blinded for viral culture experiments. 50 µL aliquots of samples VC1-VC20 were separately added to 2.4 x 10⁵ Vero E6 cells (Cell Bank, Sir William Dunn

School of Pathology, University of Oxford) in 24 well plates. Cells were propagated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS). Virus growth assays were done in DMEM supplemented with 1% FBS, glutamine and penicillin/streptomycin, according to published methods⁴². In parallel, wells of the same number of cells were cultured in triplicate without virus challenge but with 50 µL control serum (VC21), or in duplicate with a stock of Victoria/01/2020 SARS-CoV-2 passage 4 (Oxford) at calculated ten-fold serial dilutions per well of 78, 7.8, 0.78 and 0.078 plaque forming units (pfu) in 50 µL of control serum (VC21).

Wells were observed daily for cytopathic effects (CPE), using images to record all cultures on days 3 and 7 (electronically archived using [LabArchives Research Notebook](#)). We took 50 µL samples for vRNA extraction on day 3 post-challenge. Where residual sample volumes permitted, 50 µL aliquots of the respective serum were processed in parallel. In addition, 1 × 10⁸ vRNA copies produced by *in vitro* transcription and quantified by droplet digital PCR were spiked into two equivalent control media samples, and processed in parallel, to provide quantification and estimate the loss of vRNA during extraction. All samples were processed for vRNA using QIAamp Viral RNA Mini kits according to the manufacturer's instructions. RNA extracts were analysed by qRT-PCR, and vRNA copy number was interpolated from the standard curve of Ct value by known copy number. On day 4, 50 µL aliquots of supernatants from cells challenged with VC01-20 were "blind passaged" to fresh cells, and the remaining supernatants were harvested and stored separately at -80C for future analysis. After a further 3 days, we recorded CPE, if any, for second passage cultures.

RT-PCR of culture supernatant. To determine whether there had been productive infection of cells *in vitro*, we took aliquots of culture supernatant, including positive and negative controls, and serum samples for qRT-PCR analysis using CDC NP1, CDC NP2 and HKU ORF1b diagnostic panels.

For the CDC NP1 and NP2 assay, we used an N-gene digital droplet quantified *in vitro* transcribed RNA standard (GenExpress, Germany). For the HKU ORF1b assay, we used qRT-PCR quantified RNA extracts of Victoria/01/2020 SARS-CoV-2 passage 4 (Oxford) as RNA standard, diluted to 10⁷ copies/reaction and preparing a serial dilution. We used Luna Universal Probe One-step qRT-PCR kit (New England Biolabs, USA) for all reactions following the manufacturer's instructions, with either 5 µL or 2.5 µL RNA sample in a 20 µL or 10 µL reaction (see Table in Extended Data File 2⁹). Primer and probe concentrations in reactions were as specified by guidelines from the CDC and University of Hong Kong for their respective assays. PCR cycle conditions were: 10 minutes at 55°C, 1 minute at 95°C, followed by 45 cycles of 5 seconds at 95°C and 30 seconds 55°C. We manually adjusted the threshold for all runs to 0.2 and qRT-PCR efficiency was calculated for quality control. We used slopes from RNA standard curves to interpolate vRNA copy numbers in samples. Samples were analysed in six qRT-PCR runs in total. Each

qRT-PCR run contained a freshly prepared RNA standard dilution with a quantitative logarithmic range from 10^7 to 10^1 vRNA copies/reaction to calculate vRNA copy numbers across all samples.

Serological testing of serum samples for antibody to SARS-CoV-2

For a subset of clinical samples collected in Oxford (n=160), we determined an antibody titre using the Siemens SARS-CoV-2 Total (COV2T), Atellica Solution immunoassay analyser, at Public Health England Porton Down. This assay measures total antibody to Spike protein S1 Receptor Binding Domain (RBD). In a recent evaluation of performance, undertaken collaboratively by Public Health England and Oxford University, this assay had a sensitivity of 98.1% (95% CI 96.6, 99.1) and specificity 99.9% (95% CI 99.4, 100), making it the best performing of the commercially available platforms assessed⁴³. The assay was undertaken at Public Health England, Porton Down, according to the manufacturer's instructions, with a threshold of ≥ 1.0 standard units for calling a positive test.

Ethics

Acute hospital in-patients were recruited into the Sepsis Immunomics study (Ref: 19/SC/0296). Convalescent healthcare workers with hospital encounters (n=38) and convalescent patients (n=32) provided informed consent for recruitment into the ISARIC WHO Clinical Characterisation Protocol UK (ISARIC WHO CCP-UK), with ethics approval by the South Central (Oxford C) Research Ethics Committee in England (Ref: 13/SC/0149), and Scotland A Research Ethics Committee in Scotland (Ref: 20/SS/0028). Additional convalescent healthcare workers were recruited by the Oxford GI Biobank, n=3 (approval by Yorkshire and The Humber - Sheffield Research Ethics Committee, ref. 16/YH/0247). Healthy pre-pandemic control samples were used under NHSBT ethics, providing donor consent for their anonymised samples to be used in research.

Statistical analysis

Anonymised data were stored using Microsoft Excel. We analysed and presented data using [GraphPad Prism](#) v.8.3.1. Statistical analyses were undertaken using [R](#) 3.6.2. Binomial confidence intervals are presented for proportions. Univariable and multivariable logistic regression models were used to determine associations between detectable vRNA and time since symptom onset, disease severity and patient sex and age, accounting for any non-linear effects of continuous factors using natural cubic splines. Meta-analysis was undertaken using the meta package for R, version 4.12.

Results

Literature review to determine the frequency and clinical associations of RNA-aemia

We identified 28 relevant studies (Table 1; Table S1), among which 22 contained metadata suitable for meta-analysis (Figure 1). Point estimates for the frequency of vRNA detection are presented for each study representing ≥ 5 individuals, together with 95% confidence intervals (Figure 2; Table S1). We observed

considerable heterogeneity in the range of estimates for vRNA-aemia, from 0% in several studies^{21,23,25,26,37}, up to 76% in a report of patients in a critical care setting¹⁸. Pooling the data from these reports, the point estimate for the prevalence of vRNA in blood products in the 28 days following symptomatic infection is 10% (95%CI 5-18%, random effects model).

Viral RNA-aemia was reported in association with more severe disease in some studies, including a higher risk of admission to critical care settings, and increased incidence of acute clinical deterioration^{14,16,17,27}. One study reported lower RNA levels in serum compared to other sample sites³, whilst another found RNA levels in blood to be no different to that of other sample types⁴. In a small number of reports that included specific Ct values, these were typically high, although studies used variable PCR targets and different thresholds for reporting positivity (details of methods and reported Ct values are available in Table S2).

We excluded two studies from the meta-analysis because they focused on cohorts with different characteristics from all other sample sets. One of these reported PCR results from samples taken at timepoints beyond 28 days, among which none contained vRNA³⁷. The other investigated vRNA-aemia in healthy blood donors in Wuhan, China at the time of the peak of the local epidemic in the first three months of 2020, finding vRNA in six samples from among >7000 screened³⁸.

Frequency and timing of SARS-CoV-2 RNA-aemia in a local cohort

Our local clinical sample set included n=212 samples from 167 patients (median age 57 years, IQR 46-76), 89 male (53%). In 163 patients for whom clinical data were available, disease was classified as asymptomatic (n=1, 0.6%), mild (n=81, 50.0%), severe (n=37, 22.7%), or critical (n=44, 27.0%). In this sample set, collected at a median of 11 days post symptom onset (IQR 7-17 days), 27/212 were PCR positive for vRNA (12.7%, 95%CI 8.6-18.0%). Deduplicating this to represent 167 unique individuals, 20 (12.0%, 95%CI 7.5-17.9%) had RT-PCR positive serum at any time point tested. Considering all 212 samples in a multivariable analysis, critical disease severity was associated with increased vRNA-aemia, comparing mild and asymptomatic cases to severe (OR 2.3, 95%CI 0.5-12.6, p=0.29) and critical cases (OR 7.5, 95%CI 2.0-37.3, p=0.006) (Table 2). Within this dataset there was moderate statistical evidence of a trend towards decreased odds of vRNA-aemia over time (OR, per day, 0.95, 95%CI 0.89-1.00, p=0.12) (Table 2; Figure 3).

Pooling our hospital data with results from the NHSBT convalescent cohort, vRNA was detected in 23/131 (17.6%, 95%CI 11.5-25.2) samples collected up to day 13, 4/40 samples from between day 14-27 (10.0%, 95%CI 2.8-23.7%), and 0/494 samples at ≥ 28 days (0%, 95%CI 0.0-0.7%) (Figure 2B). Day 20 was the latest time point at which any PCR positive sample was collected.

Ct values for all of our 27 PCR-positive sera were high (median 40.9, range 33.6-44.8). Using the more stringent Ct threshold of 37 that may be applied by clinical laboratories to report

Table 2. Odds ratios (OR) for associations between RNA-aemia and other patient characteristics, among 212 adults with confirmed COVID-19 infection recruited at Oxford University Hospitals NHS Foundation Trust.

Clinical attribute	Multivariable OR	95% CI		P value
Age, per 10 years	0.97	0.74	1.29	0.85
Sex, Female	1.00	<i>(reference)</i>		
Sex, Male	1.54	0.61	4.10	0.37
Severity, Mild or Asymptomatic	1.00	<i>(reference)</i>		
Severity, Severe	2.31	0.51	12.64	0.29
Severity, Critical	7.46	2.02	37.31	0.006
Time from symptom onset, per day	0.95	0.89	1.00	0.12

a positive result, only 7/25 fell below this cut off, reducing our overall positive rate to 7/212 among the local clinical cohort (3.3%, 95%CI 1.3-6.6%) or 7/674 across our entire sample set (1.04%, 95%CI 0.42-2.13%).

Cytopathic effects arise in cell cultures inoculated with reference viral stock, but not in samples from COVID-19 patients or pre-pandemic controls

Healthy uninfected control cell cultures of Vero E6 cells were established (Figure 4A). We observed substantial cytopathic effects (CPE) in all samples inoculated with reference virus, characterised by cell rounding up and detaching (Figure 4B). CPE of this type was observed in wells challenged with 78 and 7.8 pfu, and moderate but typical CPE was observed in one well challenged with calculated 0.78 pfu reference virus. Cells exposed to a 1/10 dilution of control plasma did not show typical viral CPE. However in contrast to the CPE seen with reference virus, these control samples, the VC01-20 test cultures, and the culture inoculated with 0.078 pfu, instead showed variable

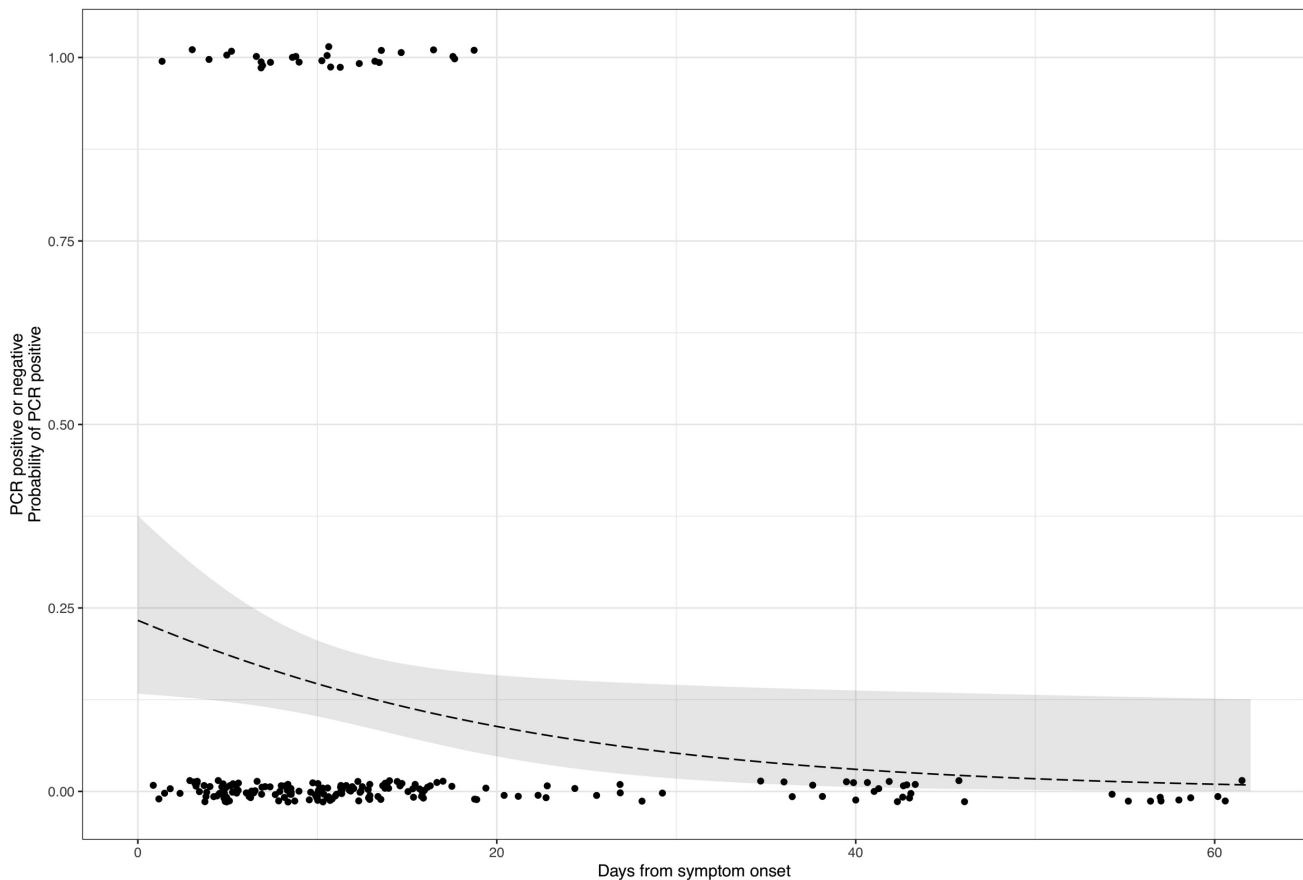


Figure 3. Relationship between RNA-aemia and days from COVID-19 symptom onset. Data shown for 212 samples collected from acute and convalescent adults from the Oxford University Hospitals cohort. Positive and negative results are shown plotted at 1 and 0 on the y-axis respectively, with jitter applied to show all points. The line shows the univariable predicted probability of RNA detection over time (95% CI: shaded).

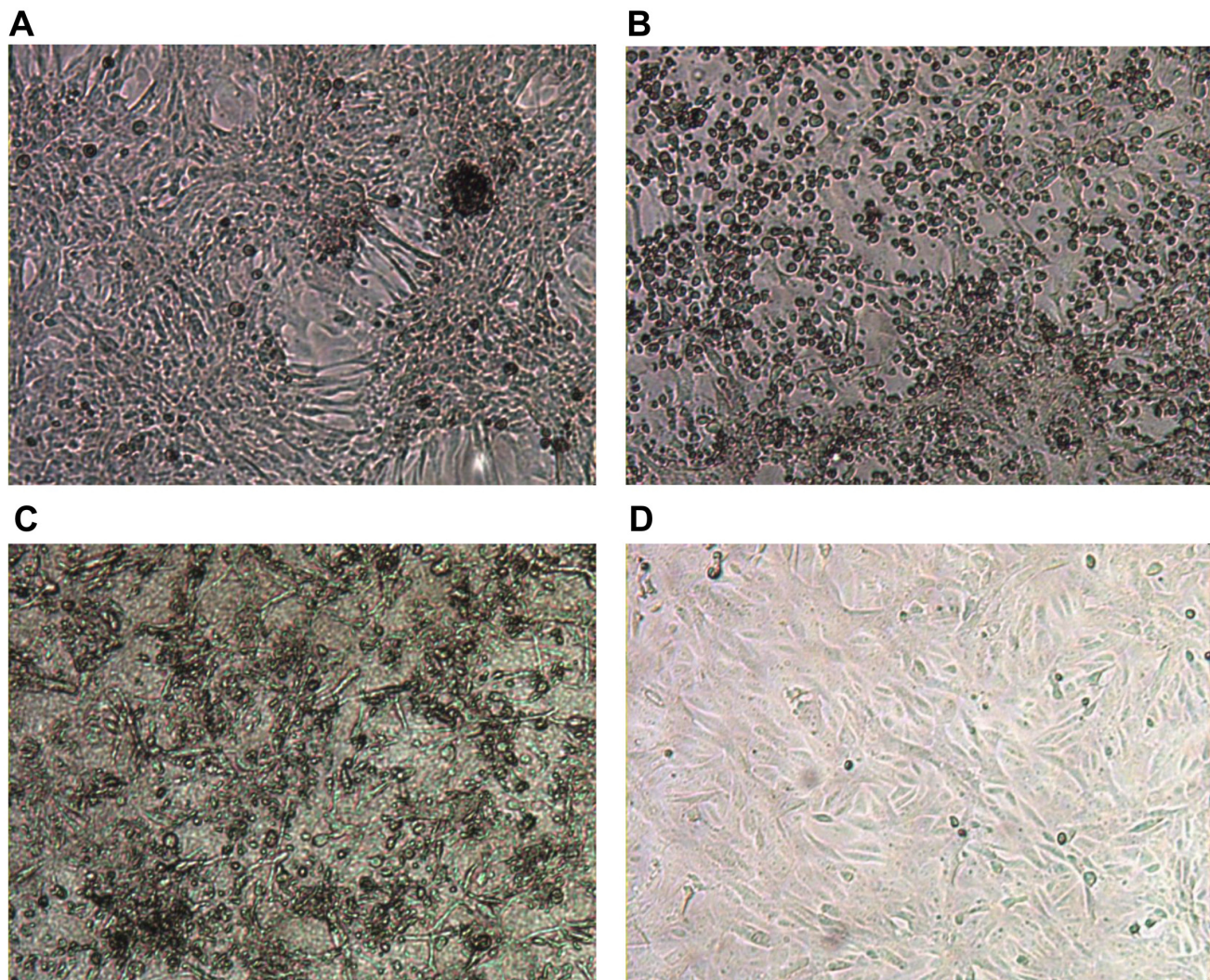


Figure 4. Typical images from cell culture in an *in vitro* system for SARS-CoV-2 culture. Top row shows controls: (A) Negative control Vero E6 cells in media; (B) Cytopathic effect (CPE) in Vero E6 cells spiked with Victoria/01/2020 SARS-CoV-2; Bottom row shows Vero E6 cells inoculated with 1/10 dilution of serum sample from sample VC12 (patient ID UKCOV040), that tested positive for SARS-CoV-2 RNA by RT-PCR; (C) Aberrant cellular effects at day 4 in a culture inoculated with VC12 at day 0; (D) Normal appearance of cells at day 7 inoculated with 1/10 dilution of the culture supernatant of the VC12-challenged culture, illustrated in (C). Raw unedited microscope images can be accessed individually on line⁹.

cellular abnormalities and noticeable gel-formation in most samples (Figure 4C). Second passage cultures were undertaken in all cases, and none showed evident cytopathic effects at day 7 (Figure 4D). This approach is limited to the sample volume of 50 μ L but we have demonstrated a single pfu at this volume reliably. Our detection threshold is <20 pfu/mL plasma, suggesting that plasma samples contain <0.1 infectious unit per 50 μ L.

RT-PCR of culture supernatant

To determine whether there had been productive infection of cells *in vitro*, we took aliquots of culture supernatant for RT-PCR. From the positive control cultures, any culture receiving ≥ 1

infectious unit of virus (78 and 7.8 pfu in 50 μ L of control serum) on day 0 produced $\geq 1 \times 10^5$ copies of viral RNA (vRNA) per sample by day 3, detected by all three (CDC NP1, CDC NP2 and HKU ORF1b) primer/probe sets. All diagnostic panels also detected low levels of vRNA in the culture inoculated with a calculated dose of 0.078 pfu. These vRNA traces are likely to reflect fragments and RNA debris from the cells in which the virus was grown.

No serum sample, and no serum-inoculated cultures had >100 vRNA copies by day 3 based on the CDC, NP1 and NP2 assays. Marginal vRNA was detected in 10 serum samples, but none

of these showed a rising titre by day 3 and none had vRNA levels within the reliably quantifiable range. The highest were in the range found in the sub-infectious dose positive control cultures (Extended Data File 2⁹). In contrast, no vRNA copies could be detected in serum-inoculated cultures tested at day 3 using the HKU ORF1b primer/probe set, while for the majority of original sera samples the HKU ORF1b assay gave similar results to the CDC, NP1 and NP2 diagnostic panel. The only exception from this was VC15, where marginal vRNA was detected in both serum and serum-inoculated cultures. These results suggest that no rising titre or no vRNA can be detected in serum-inoculated cultures. The comparison between the CDC and HKU diagnostic panels highlights interesting differences for detection of SARS-CoV-2 virus and should be explored further.

Relationship between serum SARS-COV-2 RT-PCR and antibody titre

We derived SARS-CoV-2 antibody titres for 160 clinical samples using a commercial immunoassay (Siemens); raw data are available in Underlying Data File 1. The antibody test was positive in 100/160 (62.5%) of serum samples tested, and the rate of positivity was higher in PCR-negative compared to PCR-positive samples ($p=0.019$; Figure 5A). However, there was no quantitative difference in antibody titres according to the PCR-status of the sample ($p=0.14$; Figure 5B). Among 23 PCR-positive serum samples, antibody titres varied across the dynamic range of the Siemens assay; in this small sample set, the absence of CPE *in vitro* was not dependent on the detection of total antibody (Figure 5C).

Discussion

Impact of results

Recognition that SARS-CoV-2 RNA may be detected and quantified in blood highlights its potential provenance as a biomarker, but also raises concerns about safety for personnel handling samples in clinical and research environments. Protocols

to underpin the safe handling of blood samples need to consider the best evidence for routes and risks of transmission in order to mandate safe laboratory practice, being informed by the nature of the samples and the specific task being undertaken (including any risk of aerosol generation), while also maintaining optimum cost effective workflow of clinical samples. Local risk assessments may currently result in disparate protocols being established by different organisations, but risk assessments should be proportionate, and - as far as possible - unified, and evidence-based. Developing new data to support laboratory practice is an important foundation for standardising practical guidelines.

Based on a systematic review of the literature, together with our own data, we estimate that SARS-CoV-2 RNA may be present at low copy numbers in ~10% of blood samples obtained from individuals with COVID-19 prior to day 28, most of which arise at earlier timepoints and in the setting of more severe disease. Despite being PCR-positive for vRNA, none of our clinical samples exceeded the threshold for viral infectivity.

Relationship between viral load and disease phenotype

The Ct values reported in the literature and in our local samples are high, reflecting low copy numbers and suggesting that assays may be detecting genomic fragments rather than replication-competent virus in blood. However, it is also possible that intact virions are present, but that these are immune-complexed or otherwise neutralised, accounting for the lack of CPE in our culture system.

A previous study reported a decline in RNA-aemia in severe cases from 45% at the time of admission to 11% by week 4, and in mild cases from 27% to 0% over the same time period, although these differences did not reach statistical significance³. Viral load (measured by qRT-PCR) in respiratory samples has been correlated with disease severity^{3,44,45}. Detection of vRNA in blood therefore may be more common in severe/critical disease as

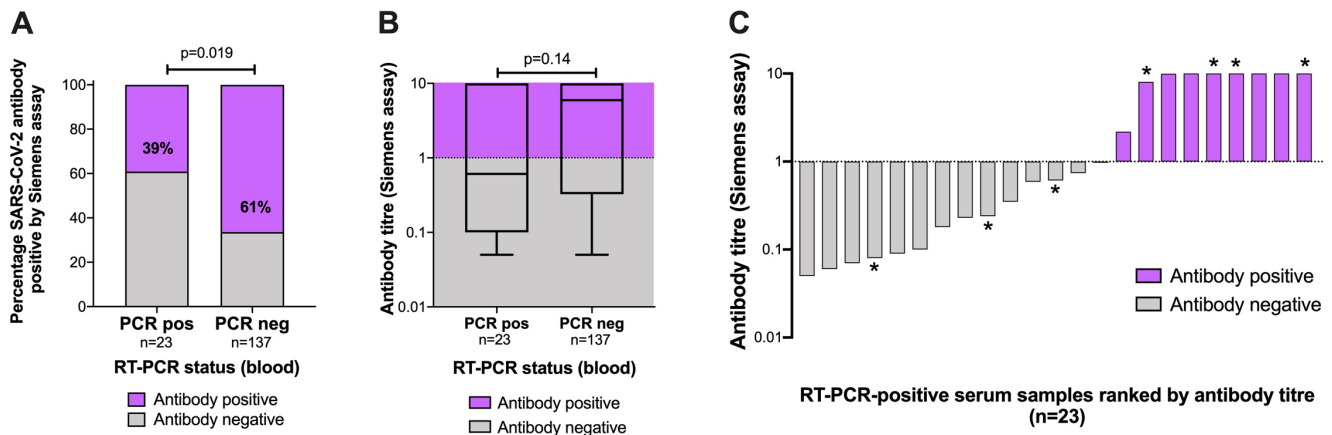


Figure 5. Relationship between serum SARS-COV-2 RT-PCR and total antibody titre, determined by Siemens SARS-CoV-2 Atellica assay. (A) Proportion of samples testing antibody positive according to RT-PCR status of serum sample; p-value by Fisher's Exact Test; (B) Distribution of IgG titres in samples according to RT-PCR status of serum sample; boxes show median/IQR and whiskers show range; p-value by Mann-Whitney U test; (C) Antibody titres in 23 serum samples testing RT-PCR positive for SARS-CoV-2 RNA, presented in rank order. The bars marked with an asterisk indicate the samples for which *in vitro* culture was attempted. In all panels, antibody positivity is reported according to the threshold set by the assay manufacturer.

a result of higher viral loads overall, or specifically relating to a high burden of infection in the lungs leading to spill-over into the circulation, or reflecting the destruction of infected cells in the respiratory epithelium. Multisystem end-organ disease caused by SARS-CoV-2 could reflect systemic viral dissemination by blood or lymphatics (potentially with direct infection of lymphocytes), or may arise as a consequence of a sepsis syndrome triggered primarily by localised pulmonary infection⁴⁶. Given the high Ct values for vRNA in blood, the identification of virus in the vascular compartment currently remains non-specific; further work is needed to understand its origins and significance, and to determine whether vRNA in the blood is innocuous or could contribute to immune dysfunction and the systemic inflammatory process.

Further work is needed to determine the bioburden and clinical significance of SARS-CoV-2 in other tissue types, for example in faeces^{47,48}. Different clinical and laboratory infection control practices should be considered for specific sample types, ideally based on an understanding of the frequency and duration of carriage and assessment of whether infectious virus can be detected.

Relationship between antibody detection and RNA-aemia

The relationship between RNA-aemia and antibody status may be a causal one, in which neutralising antibodies directly reduce viral titres in blood. However, this association may also be a feature of the time-course of evolving infection, as samples collected early in infection are both more likely to contain a 'spill over' of virus from the respiratory tract into blood, and to be seronegative, reflecting a 'window period' before seroconversion⁶. We have not tested neutralising activity on this current sample set, but a number of studies now show a close association between IgG titres and neutralisation, which confirms the biological activity of the antibodies *in vitro*^{49,50}, suggesting a causal association is entirely plausible. It should also be noted that commercially available platforms for antibody testing are validated on the basis of reporting a binary read-out, based on positive/negative thresholds set by the manufacturer, but neutralising antibodies may be present even below this threshold which could explain the lack of CPE even in samples for which the Siemens assay was reported as negative.

Caveats and limitations

Datasets reported in the literature represent mostly a small number of carefully selected patients, typically in the acute hospital setting and therefore biased towards inclusion of more unwell patients meeting WHO criteria for severe or critical disease. Recognising that the field that is currently moving at pace, we elected to include papers from the pre-print server MedRxiv, for which peer review has not been undertaken. As a result, not all material included has undergone this quality assurance step. Published reports frequently do not include timing of sample collection relative to diagnostic respiratory samples and/or symptom onset, samples from individuals with trivial or absent symptoms are not well represented in existing studies, and there are insufficient data to distinguish between frequency or quantification of vRNA present in whole blood, versus serum or plasma.

Due to the logistics of rapid recruitment of different patient groups through different pathways, RT-PCR methods varied by cohort, potentially introducing some variation in the sensitivity of detection. In our clinical samples, we adopted an inclusive approach to reporting detection of vRNA, by including samples with Ct values above those which would normally be called positive by a clinical diagnostic facility. This may lead to an over-estimation of the true prevalence of RNA-aemia in this sample group. Many previous publications do not report Ct values and direct comparisons between datasets are therefore difficult.

The absence of CPE and amplification of vRNA must be considered within the constraints of the low sample volume (50 µL in each assay), and the limits of detection within the assays used. We tested serum samples after they had been subjected to a freeze/thaw cycle, which could also have potential influence on retrieval of infectious virus. However, as samples were frozen in accordance with standard laboratory operating protocols within a few hours of collection, we anticipate this would have a limited impact on viral replication capacity, as has been demonstrated previously for other viruses⁵¹⁻⁵³.

Conclusions

Our data confirm that blood from COVID-19 patients may contain detectable RNA, but this arises in a minority of samples and is typically in low copy numbers, often outside the threshold that would be reported as positive in a clinical diagnostic laboratory. Based on evaluation of a small sample set, we have found no evidence to suggest that blood samples containing RNA could yield replication competent virus, suggesting a negligible risk of transmission of SARS-CoV-2 to healthcare workers and laboratory staff from handling such material. However, laboratory practice should be informed by guidance from Public Health England⁵⁴, CDC⁵⁵ and WHO⁵⁶; individual risk assessment is important to account for the nature of the material being handled and the process being undertaken. Universal precautions and routine safety procedures should be carefully observed, not only to protect from SARS-CoV-2 infection but also to provide protection from other potential pathogens. Further data are needed to determine the extent to which serum PCR positivity for vRNA is useful as a diagnostic or prognostic marker in patients with COVID-19 infection.

Data availability

Underlying data

Figshare: SARS-CoV-2 RNA in blood. <https://doi.org/10.6084/m9.figshare.12278249.v8>.

This project contains the following underlying data:

- Underlying Data File 1.xlsx (Metadata table for serum samples from adults with confirmed SARS-CoV-2 infection, based on RT-PCR nose/throat swab and/or EuroImmun antibody titre)

Sheet 1: samples obtained through patients recruited into a UK clinical cohort at Oxford University Hospitals NHS Foundation Trust (n=212 samples from 167 unique individuals). Cells highlighted in blue show follow-up samples collected

from the same individual at different time points. Cells highlighted in orange show serum PCR positives. All individuals had a diagnosis based on an RT-PCR throat swab positive for SARS-CoV-2. Sheet 2: samples obtained from convalescent donors a minimum of 28 days post resolution of symptoms, via NHS Blood and Transplant, NHSBT (n=142 samples from 142 individuals).

- Fig 4A 20200501_cc1.jpg (raw unedited microscope images for [Figure 4A](#))
- Fig 4B 20200501_lin100_2.jpg (raw unedited microscope images for [Figure 4B](#))
- Fig 4C 20200501_vc12.jpg (raw unedited microscope images for [Figure 4C](#))
- Fig 4D 20200504_vc12.jpg (raw unedited microscope images for [Figure 4D](#))

Extended data

Figshare: SARS-CoV-2 RNA in blood. <https://doi.org/10.6084/m9.figshare.12278249.v8>.

This project contains the following extended data:

- Extended Data File 1.xlsx (Metadata table providing data for prevalence of SARS-CoV-2 RNA in blood and blood products based on a systematic literature review. Details of 28 citations are presented, and the 22 studies included in quantitative meta-analysis are indicated)

- Extended Data File 2.pdf (qRT-PCR quantification of vRNA from sera and viral culture assays. Calculation of vRNA copy numbers, and qRT-PCR results in figure and table format.)
- RT-PCR Primer sequences.xlsx (Primer sequences)

Reporting guidelines

Figshare: PRISMA and STROBE checklists for 'SARS-CoV-2 RNA detected in blood products from patients with COVID-19 is not associated with infectious virus' <https://doi.org/10.6084/m9.figshare.12278249.v8>.

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Since SARS-CoV-2 is a RNA virus, the sample pre-treatment is very critical to the RNA stability and virus viability. So 1) please give the details of the serum preparation protocol including the types of tubes, the duration prior to serum preparation, any heat-inactivation, et al. 2) should try to spike the RNA standards into normal human whole blood to assess the damage of serum preparation to RNA if possible. 3) Please state what is the SARS-CoV-2 antigen that was used for Siemen's antibody detection kit. Correlations between neutralizing activities with antibodies titers to N protein, RBD, S1, and S will be very different.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: antibody drug, COVID-19 vaccine development and diagnostic tools for cancers and infectious diseases, contract antibody and assay development.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 15 October 2020

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William L. Irving 

NIHR Nottingham Biomedical Research Centre, Nottingham University Hospitals NHS Trust, Nottingham, UK

Thank you for the update on this article. I am impressed that the team have been able to conduct additional studies on the relationship between antibody status and RNA detection, which I think adds to the value of the article.

I have no further comments.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Clinical virology; pathogenesis, epidemiology and treatment of viral hepatitis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 24 August 2020

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**William L. Irving**

NIHR Nottingham Biomedical Research Centre, Nottingham University Hospitals NHS Trust, Nottingham, UK

- This work sets out to determine the frequency and significance of detection of SARS-CoV2 RNA in blood. This is a pertinent and relevant question to address. The methods are well described in great detail.
- The literature review yielded a point estimate for the prevalence of vRNA in blood products in the 28 days following symptomatic infection of 10% (95%CI 5-18%). Copy number in positive samples was uniformly low. When testing local samples, 20/167 (12%) patients had RT-PCR positive samples at any time point tested. vRNAemia levels tended to decrease over time since onset of symptoms/diagnosis, and also correlated with critical disease severity.
- Attempts to culture virus from RT-PCR positive samples were unsuccessful, suggesting the virus was not replication competent.
- In the discussion, the sentence "Different clinical and laboratory infection control practices to be considered for specific sample types, to determine the frequency and duration of carriage and to assess whether infectious virus can be detected." isn't really a sentence.
- This is an excellent study providing valuable data which will contribute to our understanding of the significance of detection of RNA-aemia.
- My one substantive comment relates to possible neutralisation of virus present in the bloodstream by antibodies. I don't think (apologies if I've missed it) this is mentioned in the discussion. There is considerable patient-to-patient variation in generation of Nabs. It would be of great interest to know the frequency and titre of neutralising antibody in each of the sera tested. This would allow (i) assessment of whether detectable RNA correlates (most likely inversely) with the presence/titre of Nab, and (ii) the selection of RNA positive sera with the lowest Nab titre for virus culture, to address the hypothesis that failure to culture

virus may be linked to the presence of high titres of Nab. If it is not practicable to do this, then I think it would be worth adding a sentence or two to the discussion to raise this possibility.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Clinical virology; pathogenesis, epidemiology and treatment of viral hepatitis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 29 Sep 2020

Philippa Matthews, Oxford University Hospitals NHS Foundation Trust, John Radcliffe Hospital, Headington, Oxford, UK

Thank you to the reviewer for the helpful feedback and positive comments.

With regard to the error in the discussion, we have corrected the sentence that contained grammatical errors to read as follows: 'Different clinical and laboratory infection control practices should be considered for specific sample types, ideally based on an understanding of the frequency and duration of carriage and assessment of whether infectious virus can be detected'.

The question about antibody detection in our sample set is a very good one. We have been able to add data for antibody titres in a subset of our samples (n=160), determined by the Siemens immunoassay, a validated commercial platform. We have added the raw data to an updated supplementary metadata table on-line, and have generated a new figure

presenting these results (Fig 5). Our analysis shows that, as expected, the samples that are PCR-positive were significantly less likely to be antibody positive ($p=0.019$; panel A). However, there was no significant difference in quantitative antibody titres between PCR-positive and negative samples ($p=0.2$, panel B). Presenting the antibody titres in PCR-positive sera shows no clear cut-off in IgG titre that is associated with PCR-positivity (panel C), and demonstrates that positive antibody status was not pre-requisite to preventing CPE *in vitro*. We have added to the discussion to point out that we cannot determine a causal relationship between the development of antibody and loss of detectable RNA in samples, particularly as we have not specifically measured neutralising activity in this sample set, but there are emerging data from other sources to support a correlation between titres and *in vitro* neutralisation. We have modified the methods section to incorporate description of the Siemens assay, updated the results to present these data, and added a short paragraph to the discussion to provide context and interpretation. The antibody data are now also referenced in the results section of the abstract.

Competing Interests: N/A
