Shedding of culturable virus, seroconversion, and 6-month follow-up antibody responses in the first 14 confirmed cases of COVID-19 in the United States

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Abstract

We aimed to characterize presence of culturable virus in clinical specimens during acute illness, and antibody kinetics up to six months post-onset, among 14 early US COVID-19 patients. We isolated viable SARS-CoV-2 from rRT-PCR-positive respiratory specimens collected during days 0-8 post-onset, but not after. All 13 patients with two or more serum specimens developed anti-spike antibodies; 12 developed detectable neutralizing antibodies. We did not isolate virus after detection of neutralizing antibodies. Eight participants provided serum at six months post-onset; all retained detectable anti-spike IgG, and half had detectable neutralizing antibodies. Two participants reported not feeling fully recovered at six months.

Key Words: SARS-CoV-2, COVID-19
Introduction

Since identification of the first confirmed COVID-19 cases, numerous studies have described a high percentage of seroconverting patients, with detection of viral RNA in respiratory and non-respiratory specimens even after resolution of symptoms[1–4]. However, the duration and peak of infectiousness, is still not fully understood, though has been reported to peak around symptom onset [5]

Given the short time frame of SARS-CoV-2 circulation, little is known about the longevity of immune response. Recent studies have indicated waning of serum antibodies by 4 months post-onset among mildly symptomatic and asymptomatic convalescent patients[6, 7] while others have indicated persistence of pan-immunoglobulin antibodies and neutralizing antibodies at 4–5 months [8, 9]. Furthermore, the persistence of symptoms and possible longer-term effects of SARS-CoV-2 infection need further characterization [10]. In this investigation, we sought to characterize the ability to isolate virus from patient samples from the first 14 reported symptomatic U.S. COVID-19 cases in relation to antibody seroconversion. Additionally, we examined the persistence of serum antibody responses at six months, and participant health and healthcare use in the six months since illness.

Methods

Specimen collection and human subjects research

Identification, initial interview, and specimen collection for patients A–L were described previously[1]. Patients M and N were identified shortly after these cases through screening of repatriated individuals returning from Wuhan, China. Patients were contacted by their local or state health department to participate in voluntary 6-month follow-up blood collection and interview and were excluded if they were no longer residing in the United States. Participants
were interviewed about their health and healthcare use since their COVID-19 illness using structured and open-ended questions. This activity was reviewed by CDC and was conducted consistent with applicable federal law and CDC policy.* Forms for the initial public health investigation were approved under Office of Management and Budget, number 0920-1011.

**Reverse Transcriptase Real-time PCR**

During initial investigations[1] 492 nasopharyngeal (NP) swabs, oropharyngeal (OP) swabs, sputum (if available), serum, urine and stool specimens were collected every 2–3 days and tested by reverse transcriptase real-time polymerase chain reaction (rRT-PCR) [11]. Samples were reported as either negative (no targets [nucleocapsid (N)1, N2 or N3] positive), inconclusive (1 or 2 targets positive) or positive (all 3 targets positive) [11].

**Protein expression and ELISAs**

Sera were tested for SARS-CoV-2 spike (S) pan-immunoglobulin (Ig), IgG, IgM, and IgA by enzyme-linked immunosorbent assay (ELISA) using the ectodomain of pre-fusion stabilized S1/S2 (S) [12]. Briefly, plates were coated with 150 ng/ml S and blocked using 2.5 x StabilCoat blocker (Surmodics). Sera were serially diluted 1:100 to 1:102,400 in serum diluent (PBS-tween/5% skim milk), incubated with HRP-conjugated goat anti-human antibodies (SeracareKPL) diluted to1:2000, and developed with ABTS peroxidase substrate system (SeracareKPL). Plates were read at 405 and 490 nm. Final background corrected ODs were calculated as the difference in absorption (490-405; antigen-coated – PBS-coated well).

**Cell culture, viral isolation, and microneutralizations**

To characterize recovery of viable virus, available rRT-PCR positive and inconclusive specimens collected during days 0-29 post illness onset were used for viral culture (Supplemental Fig 1). Prior to virus culture, swabs in viral transport media, stool, serum and
sputum sere stored at -80°C. Patient specimens were thawed, diluted serially in DMEM-dilution and Vero CCL-81 cells were innoculated [13]. Cultures showing evidence of cytopathic effect (CPE) were tested by SARS-CoV-2 rRT-PCR and viral recovery was defined as successful if the first viral passage had an N1 Ct at least 2 lower than the clinical specimen. After confirmation, virus propagation continued to generate laboratory stocks.

Before microneutralization, sera were heat inactivated for 30 minutes at 56°C. Sera were diluted 2-fold in DMEM and 100 TCID₅₀ per well SARS-CoV-2 WA_1/USA/2020 were added to diluted sera in triplicate at 1:20-1:640. Freshly trypsinized Vero CCL-81 were added to diluted sera and virus and incubated for 5 days. Cells were fixed and stained with crystal violet fixative. Endpoint titers was defined as the highest dilution to block viral CPE in all 3 wells.

Data analysis

Data were analyzed using GraphPad Prism 7 and R v.3.6.2. Ct values for each of the three targets (N1, N2, and N3) were compared among respiratory specimens from which virus was recovered versus not recovered using a Mann-Whitney test. ELISA titers were calculated by performing Log₂ transformations, performing spline analysis, and extrapolating the titer at the cutoff of 0.4. Pan-Ig and neutralizing antibody titers were then compared among patients receiving and not receiving supplemental oxygen using a Mann-Whitney test. Comparisons of antibody titers were performed using Log₂ data transformation and linear regression with coefficients of determination (R²) presented as measures of correlation.

Results

All 14 patients were symptomatic, with symptom resolution among 13 patients reported (based on initial public health interview) at five–36 days after onset (median 13 days). Four patients received supplemental oxygen; one of whom required intensive care
level monitoring, none received mechanical ventilation. All patients survived. The clinical course and rRT-PCR results for 12 of the 14 patients were described previously[1].

Viral culture was attempted on 131 rRT-PCR-positive or inconclusive specimens including 57 NP swabs, 42 OP swabs, 14 sputum, 3 sera and 15 stool specimens. Successful virus culture varied by specimen type, RT-PCR cycle threshold (Ct) value and timing of collection post-onset (Supp. Figure 2). Virus was recovered from 14 respiratory specimens collected during days zero-eight post-onset, including 25% 4/16 rRT-PCR-positive specimens collected during days 0–2 post-onset, 29% (5/17) collected during days 3–5 post-onset, and 24% (5/21) collected during days 6–8 post-onset. Successful culture was observed in 14% (8/57) NP swabs, 10% (4/42) OP swabs, and 14% (2/14) sputum specimens with nucleocapsid (N)1, N2, and N3 Ct values ranging from 16.5–32.5, 17.7–32.6, and 16.7–31.4 respectively (Supplementary Figure 1). N1, N2, and N3 Ct values were all significantly lower (p<0.0001 for all 3 targets) among specimens from which virus was recovered versus not (Supplementary Figure 2). We did not recover virus from stool, any specimens collected nine–29 days post symptom onset, from rRT-PCR-positive serum specimens or from any inconclusive specimens (Supplementary Figure 3). All urine specimens were negative by rRT-PCR.

Successful virus culture was observed in four of four (100%) patients who received supplemental oxygen and five of 10 (50%) patients who did not (Figure 1). Three patients received Remdesivir. Two patients (B & L) had negative viral culture results before Remdesivir treatment was initiated. In one patient (C), Remdesivir was started on day 10 after illness onset, and virus culture on a respiratory tract specimen collected that day was negative (Figure 1).
We tested serum specimens from 14 patients collected 0–42 days post symptom onset for antibodies; 13 had at least two acute sera available to assess antibody kinetics. All 13 demonstrated seroconversion (i.e. detectable pan-Ig antibodies) during days three–21 (median 8 days) (Figure 2A). One patient did not develop detectable IgM and two did not develop detectable IgA. Twelve patients had neutralizing antibody titers above the level of detection (titers ≥80). The patient who did not have detectable neutralizing antibodies reported only a cough, with viable virus detected at days zero and five post-onset. The median time to neutralizing antibody detection was 10 days post-onset (range: five–28 days) and did not appear to differ by illness severity (Figure 2B). When comparing maximum titers detected during day 7–42 post-onset in patients who did not vs. did receive supplemental oxygen, the median maximum pan-Ig titers were 5977 and 6400, respectively (Mann-Whitney test, p=0.33), and median maximum neutralizing antibody titers were 160 and 240, respectively (Mann-Whitney test, p=0.004 [Supplementary Figure 4]). When comparing isotype responses to neutralizing antibody titers, neutralizing titers correlated most closely with IgA titers ($R^2=0.75$) (Supplementary Figure 4).

We were unable to recover live virus after detection of pan-Ig serum antibodies, with one exception: patient C had live virus recovered on day 3 after symptom onset (Figure 1). Virus recovery was not successful in any patient after detection of neutralizing antibodies. SARS-CoV-2 RNA continued to be detected by rRT-PCR after detection of pan-Ig in 12 of 13 patients, with viral RNA detected for a median of 17.5 days post-onset (range six–36 days post-symptom onset, n=14).

Eight of 14 patients provided follow-up plasma samples at approximately 6 months after symptom onset (Supplementary Table 1). By six months post-onset, all eight individuals still showed detectable IgG antibodies, although titers had waned over time (Figure 2A). Five patients still demonstrated high IgG titers at six months including two of five patients who
did not receive supplemental oxygen and all three patients who received supplemental oxygen (Figure 2A). Four of eight (50%) patients had neutralizing antibody titers above the level of detection (titers ≥80) (Figure 2B). One patient had detectable IgM and two had detectable IgA. One patient (G) reported recently sharing a household for three weeks with two persons diagnosed with COVID-19; however, all household members isolated appropriately, used separate bedrooms and bathrooms, and wore masks when they interacted.

At 6 months post-symptom onset, six of eight patients reported feeling fully recovered with no ongoing medical issues (Supplementary Table 1). Two patients reported not feeling fully recovered; one reported intermittent headaches and insomnia since their illness or soon after, and another patient with underlying COPD reported feeling mostly well but with some ongoing chest congestion and cough felt to be slightly worse than before their COVID-19 illness.

**Discussion**

In summary, live virus was not detected more than 8 days post-onset, binding IgG antibodies were still detectable at six months post-onset, and neutralizing antibodies were detected in some patients at six months. These findings are consistent with those shown previously[5, 8, 9, 14]. Two of eight participants reported still not feeling fully recovered at six months post-onset. While these symptoms were self-reported, this supports the growing understanding that long term effects of SARS-CoV-2 infection warrant further investigation.

We recovered live virus from rRT-PCR-positive respiratory specimens with C_{t} values ranging from 16.5–32.6 using a non-quantitative assay, but did not recover live virus from
serum or stool specimens, from inconclusive respiratory specimens, or after symptom resolution, despite continued detection of viral RNA. $C_t$ values should not be used as direct marker of whether an individual is infectious because specimen handling can affect $C_t$ and the assay is not quantitative. Virus recovery indicates that a patient is potentially infectious and capable of transmission, but the opposite may not be true.

Anti-S serum antibodies were detected in all patients by day 21 post-onset, and as early as day three, in line with previous reports [2,3]. Detection of serum antibodies correlated with an inability to culture virus, also consistent with previous reports [15]. This finding does not indicate causation, as other immune factors could be activated at approximately the same time. Although numbers were small, maximum neutralizing titers were higher in patients who received supplemental oxygen, suggesting more severe illness may result in higher neutralizing antibody titers.

This investigation is subject to several limitations. The number of participants is limited and only eight patients agreed to participate in the six-month follow-up specimen collection and interview. Due to the small number of patients and timing of Remdesivir administration we are unable to draw conclusions about the impact of Remdesivir on clearance of viable virus. Disease severity metrics were limited to supplemental oxygen use, and findings may differ in asymptomatic or critically ill patients. As viral culture was qualitative, we could not measure viral load. Additionally, the lack of specimens collected between day 42 and six months meant it was not possible to assess antibody decay. Lastly, due to the multitude of factors resulting in an effective immune response, the presence of detectable antibodies may not equate to protection from re-infection.
These combined virologic and clinical findings can help inform isolation guidance for patients with mild to severe COVID-19 illness. Additionally, these findings from early U.S. cases provide valuable insight into the natural history of COVID-19, including the immune response following primary SARS-CoV-2 infection.

* See e.g., 45 C.F.R. part 46.102(l)(2), 21 C.F.R. part 56; 42 U.S.C. §241(d); 5 U.S.C. §552a; 44 U.S.C. §3501 et seq).
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DISCLAIMERS

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention. Names of specific vendors, manufacturers, or products are included for public health and informational purposes; inclusion does not imply endorsement of the vendors, manufacturers, or products by the Centers for Disease Control and Prevention or the U.S Department of Health and Human Services.

Author Contributions

All authors meet authorship criteria and approve of publication. M.E.K., C.M.M. and N.J.T. had full access to all data in the study and take responsibility for the integrity of data and accuracy of data analysis. C.M.M., S.A.K., A.J.H., A.F., S.I.G., J.T.W., N.J.T. and S.L. contributed to the concept and design of the acute phase investigation and laboratory testing; C.M.M., M.E.K., J.E.T., J.T.W. and N.J.T contributed to the concept and design of the 6m follow up blood collection, interview and laboratory testing. All authors contributed to the acquisition, analysis or interpretation of data. M.E.K. M.A.U.R., C.M.M, N.J.T. drafted the manuscript. All authors contributed to critical revision of the manuscript for important intellectual content. M.E.K., M.A.U.R., G.R.A, C.M.M. and N.J.T contributed to analysis and data visualization. C.M.M., A.F., A.J.H., and N.J.T. contributed to the supervision of the
acute phase investigation and laboratory testing; C.M.M. and N.J.T. supervised the 6m follow up blood collection, interview and laboratory testing.

**Competing Interests statement**

Dr. Kujawski reports personal fees from Merck & Co., Inc., outside the submitted work. The remaining authors declare no competing interests.

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**Data Availability**

Datasets may be available from the corresponding author on request.
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Figure 1. Antibody responses, rRT-PCR data, and viral culture data, by patient, in the first 14 patients with COVID-19 in the United States. Patient numbers highlighted with an * indicate that the patient received supplemental oxygen. Antibody responses are shown as blue triangles (pan-Ig ELISA titers) and black triangles (neutralizing antibody titers). The dashed horizontal line shows the limit of detection for the antibody assays, with ELISA titers <100 and neutralizing titers <80 considered not detectable. rRT-PCR data for respiratory samples on which viral culture was attempted are shown as circles for positive specimens (all three gene targets with a Ct <40) and squares for inconclusive specimens (one or two gene targets with a Ct<40); black fill or outline indicates culture negative and red denotes culture positive. A number of key dates are also denoted on each applicable graph: a green X indicates the last reported rRT-PCR test for each patient (inclusive of positive samples for which culture was not attempted); a purple arrow indicates the first date of Remdesivir administration (if applicable); and each black arrow indicates that last date of reported symptoms.

Note. For Patient I, the last rRT-PCR positive result was 36 days post-onset. For patient F, the last rRT-PCR positive result was 32 days post-onset. Patient H reported symptom resolution 36 days after symptom onset. For Patient M, the last day of symptoms was not available.

Figure 2. Kinetics of early antibody responses are shown within the first 45 days; six-month follow-up timepoints are shown after the broken X axis. A. Anti-spike pan-Ig titers vs. days post illness, for patients who did and did not received supplemental oxygen (O₂). The dotted line at 100 represents the limit of detection. B. Neutralizing antibody titers for patients who did and did not received supplemental O₂ (same timepoints). The dotted line indicates the limit of detection and the dotted line at the top of the graphs represents the maximum dilution tested.
Figure 2

A.

Pan Ig

IgM

IgG

IgA

Patient ID

Days post illness onset

Days post illness onset

Days post illness onset

Days post illness onset

No supplemental O₂

Supplemental O₂

B.

No supplemental O₂

Supplemental O₂

Endpoint filter

Endpoint filter

Days post illness onset

Days post illness onset