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Serologic Responses in Healthy Adult with SARS-CoV-2 Reinfection, Hong Kong, August 2020

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In March 2020, mild signs and symptoms of coronavirus disease developed in a healthy 33-year-old man in Hong Kong. His first infection did not produce virus neutralizing antibodies. In August, he had asymptomatic reinfection, suggesting that persons without a robust neutralizing antibody response might be at risk for reinfection.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of coronavirus disease, which has caused a pandemic in humans. Whether SARS-CoV-2 infection induces serologic immunity and the duration of that immunity is unknown. In humans, reinfection with seasonal coronaviruses occurs naturally and in experimental conditions (*1*,*2*).

Within 30 days after infection, most persons with SARS-CoV-2 begin producing antibodies against the spike and N proteins of the virus (*3*,*4*). An outbreak of SARS-CoV-2 on a fishing vessel showed that persons with prior neutralizing antibodies against SARS-CoV-2 were not reinfected (*5*). We analyzed the serologic and cytokine responses of a patient who had 2 episodes of SARS-CoV-2 infection (*6*). These findings have implications for population immunity generated from natural infection or vaccines.

On March 23, 2020, fever, headache, cough, and sore throat developed in a 33-year-old Caucasian man with no underlying conditions in Hong Kong. Six days later, the patient was admitted to the hospital with mildly elevated levels of alanine aminotransferase (73 U/L, reference range <50 U/L) and lactate dehydrogenase (236 U/L, reference range 106–218 U/L). Chest radiographs did not show any infiltrates. He tested negative for hepatitis B surface antigen and antibodies against HIV and hepatitis C virus. He had IgG against measles virus and varicella zoster virus. Symptoms resolved completely within 3 days. A sample of the patient's deep throat saliva tested positive for SARS-CoV-2 RNA by reverse transcription PCR (RT-PCR). During days 6–20 after symptom onset, the patient tested positive 7 more times; RT-PCR cycle thresholds ranged from 31 through 36 (Figure). He was isolated in the hospital until twice testing negative for SARS-CoV-2 by RT-PCR, on days 21 and 22. At a follow-up visit on day 43 (i.e., May 5, 2020), he was asymptomatic and had resumed his usual work. We took serum samples on days 10 and 43 (Figure).

On August 15, 2020, the patient returned to Hong Kong after a 1-week trip in Spain. As a part of border surveillance, he submitted a deep throat saliva sample for RT-PCR; this sample tested positive for SARS-CoV-2 RNA. He remained asymptomatic throughout his second infection. The clinical course of this second episode has been reported elsewhere (Figure) (*6*). We confirmed the previous report (*6*) that viruses from the first and second infection of this patient were phylogenetically distinct (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/26/12/20-3833-App1.pdf), demonstrating reinfection. We collected baseline serum on day 3 after detection of reinfection (day 148 after symptom onset of his first infection) to infer his probable preinfection serologic results.

The 50% plaque reduction neutralization test (*3*) and surrogate virus neutralization test (*7*) on the serum samples collected on days 10, 43, and 148 did not detect antibodies against SARS-CoV-2. ELISA showed decreasing titers of serum IgG against the spike receptor-binding domain (RBD) of SARS-CoV-2; on day 148, the patient tested negative for these antibodies (*3*). All 3 serum samples tested negative for IgM against spike RBD (Appendix Figure 2). On day 10, the patient tested negative for N-specific serum IgG by chemiluminescent microparticle immunoassay assay (Abbott, https://www.corelaboratory.abbott) and indirect microtiter plate enzyme immunoassay; he tested weakly positive on day 43 in a validated luciferase immunoprecipitation assay (*4*) (Figure). As reported previously (*6*), a strong antibody response to N protein developed by day 5 of reinfection. This

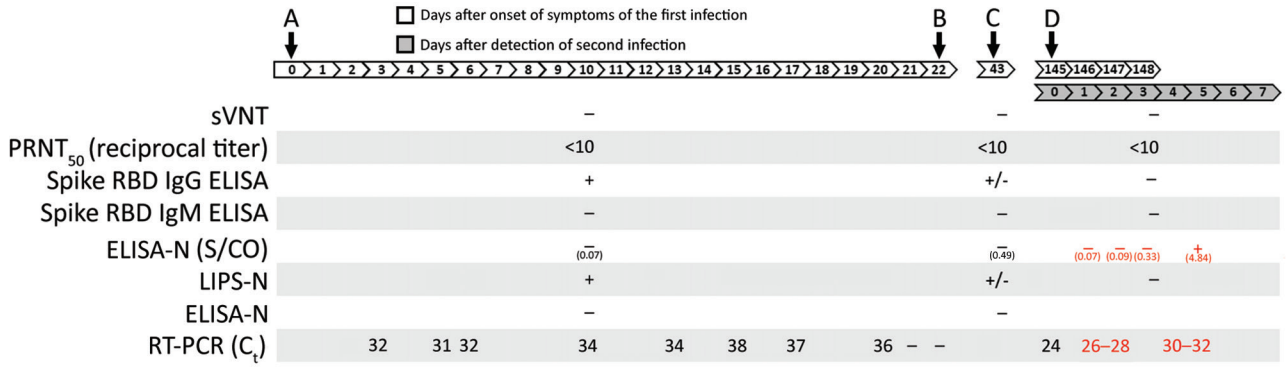

Figure. Timeline of primary infection and reinfection with severe acute respiratory syndrome coronavirus 2, Hong Kong, August, 2020. A) Onset. B) Discharge. C) Clinical follow-up. D) Mandatory testing. Black font indicates data from this investigation; red font indicates data from To et al. (*6*). C_t, cycle threshold; ELISA-N, enzyme linked immunosorbent assay for N protein; LIPS, luciferase immune precipitation assay; $PRNT_{50}$, 50% plaque reduction neutralization test titer; RBD, receptor binding domain; RT-PCR, reverse transcription PCR; S/CO, ratio of optical density readings of sample divided by cutoff (ratio of ≥1.4 considered positive); sVNT, surrogate virus neutralization test; +, positive; –, negative; +/–, borderline.

response suggests that antibody against SARS-CoV-2 developed on reinfection.

Levels of adaptive cytokine interleukin-2 were elevated on days 10 and 43 (Appendix Figure 3, panels A, B). Reinfection coincided with a stronger interleukin-21 memory type response on day 148 than on days 10 and 43.

Previous studies show that most patients with mild, severe, or asymptomatic SARS-CoV-2 infection produce neutralizing antibodies and antibodies against spike RBD and N proteins (*3*,*4*). This case was unusual because the patient had low or undetectable levels of neutralizing and binding antibodies against multiple viral proteins during his primary infection and acute stage of asymptomatic reinfection. He was not immunodeficient because he had IgG against measles and varicella zoster viruses and no history of recurrent infections. The virus from the first infection had a truncation in the 58AA open reading frame 8 gene, which mediates immune evasion through downregulation of major histocompatibility complex and interferon responses (Y. Zhang et al., unpub. data, https://www.biorxiv.org/content/10.1101/2020.05.24.111823v1) (*8*). However, it is unclear if this mutation contributed to the patient's lack of antibody production.

Reasons for this patient's unusual response need to be further investigated. He recovered from his primary infection within 3 weeks, and his secondary infection was asymptomatic. These findings indicate that, in the absence of primary neutralizing antibodies, T cells and mucosal immunity might have played a critical role in resolving the infection. Given the unusual antibody response in this patient to his first infection, researchers must be cautious about generalizing more widely from this patient's experience.

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Serologic Responses in Healthy Adult with SARS-CoV-2 Reinfection, Hong Kong, August, 2020

Appendix

Methods

Patient Follow-Up

Written, informed consent was obtained from the patient to participate in our study on COVID-19, which was approved by The Joint Chinese University of Hong Kong-New Territories East Cluster Research Ethics Committee.

Plaque Reduction Neutralization Test (PRNT)

PRNT antibodies were detected as previously described (*1*). Briefly, serial dilutions of each serum sample were incubated with 30–40 plaque-forming units of viruses for 1 h at 37°C. The virus–serum mixtures were added onto pre-formed Vero E6 cell monolayers and incubated for 1 h at 37°C in 5% CO_2 incubator. The cell monolayer was then overlaid with 1% agarose in cell culture medium. After 3 d of incubation, the plates were fixed and stained. Antibody titers were defined as the highest serum dilution that resulted in $\geq$50% ($PRNT_{50}$) reduction in the number of virus plaques.

Surrogate Virus Neutralization Assay

Neutralizing antibodies were also tested by the SARS-CoV-2 surrogate virus neutralization test kits (GeneScript USA, Inc, New Jersey) according to the manufacturer's instructions (*2*). Briefly, the test sera (60 μL), the positive and negative controls were diluted at 1:10 and mixed with an equal volume of horseradish peroxidase (HRP) conjugated SARS-CoV-2 spike receptor binding domain (RBD) protein and incubated for 30 min at 37°C. Then, 100 μL of each mix was added to the wells on the microtiter plate coated with ACE-2 receptor, the plate was sealed and incubated at 37°C for 15 min. The plates were then washed with wash-solution,

tapped dry and 100 μL of 3,3′,5,5′-Tetramethylbenzidine (TMB) solution was added to each well and incubated in the dark at room temperature for 15 min. The reaction was stopped by addition of 50 μL of Stop Solution to each well and the absorbance read at 450 nm in an ELISA reader. Assuming the positive and negative controls gave the recommended OD450 values, the % inhibition of each serum was calculated as (1 – OD value of the sample/OD value of the negative control) × 100. An inhibition of ≥20% is regarded as a positive result while that <20% is negative (*2*).

Luciferase Immunoprecipitation (LIPS) Assay

The LIPS assay was initially described by Burbelo et al. (*3*) adapted to SARS-CoV-2 by us as previously described (4). Briefly, Renilla luciferase tagged SARS-CoV-2 N antigens were produced from COS1 cells, and antigen equalized to 10^7 luciferase units for each serological test. Serum (heat inactivated and diluted 1:100 in Buffer A (50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM MgCl2, 1% Triton X-100)) was incubated with 10^7 LU of (Ruc)-N antigen for 1 h with shaking at 800 rpm. Ultralink protein A/G beads were added to the (Ruc)-antigen and serum mixture in a 96-deep-well polypropylene microtiter plate and incubated for 1 h with shaking at 800 rpm. The entire volume was then transferred into HTS plates and washed 10 times with Buffer A, and then twice with PBS. The plate was read using QUANTI-Luc Gold substrate (Invivogen, USA) as per manufacturer's instructions on a Wallac MicroBeta JET luminometer 1450 LSC & Luminescence counter and its software for analysis (PerkinElmer, USA). Experimental controls include no-serum blank wells with (Ruc)-antigens and negative control serum from age-matched noninfected patient plasma collected prior to the COVID-19 pandemic (n = 20). The background corresponds to the LU signal from each Ruc-fusion antigen with protein A/G and substrate with no serum. The cutoff limits were derived from the mean value plus 3 × SD of the negative controls.

Virus Full-Genome Sequencing

We used the methods previously described by us (*5*). Briefly, virus genome was reverse transcribed with multiple gene-specific primers targeting different regions of the viral genome. The synthesized cDNA was then subjected to multiple overlapping 2-kb PCRs for full-genome amplification. PCR amplicons obtained from the same specimen were pooled and sequenced using MiSeq sequencing platform (Illumina). Sequencing library was prepared by Nextera XT

DNA library prep Kit (Illumina) following standard protocol. Generated sequencing reads were mapped to a reference virus genome by BWA (*6*), and genome consensus was generated by Geneious version 11.1.4 (https://www.geneious.com).

Anti-SARS-CoV-2 N IgG by In-House Indirect Microtiter Plate ELISA

Purified glutathione S transferase (GST)-tagged full-length nucleocapsid fusion protein (GST-N) of SARS-CoV-2 (1 μg/mL) was coated onto Immulon 2 HB 96-well microtiter plate (ImmunoChemistry, USA) in bicarbonate buffer (pH 9.6) overnight at 4°C. Blocking buffer containing 0.25% BSA (Sigma, USA) in 1 × TBS was added into wells, followed by adding 100 μL of human plasma or serum diluted at 1:100 in 1 × TBS containing 0.2% Tween-20 at 37°C for 1 h. The plates were washed and incubated with horse-peroxidase-labelled goat antihuman IgG (Invitrogen, USA) at room temperature for 30 min. After washing, detection was carried out by adding 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Sigma, USA) in dark and the reaction was stopped by adding 2 M of sulfuric acid. The results were read at 450 nm using a VICTOR 3 Multilabel Plate Reader (PerkinElmer, USA). Mean OD of negative controls plus 3 standard deviations was defined as cut-off for reactive samples. Validation using 50 convalescent samples of patients with confirmed SARS-CoV-2 infection collected at ≥30 days from illness onset revealed 100% sensitivity, and from 50 samples taken from healthy persons before November 2019 revealed 100% specificity.

IgG Against Varicella Zoster Virus and Measles by Commercial Microtiter Plate ELISA

Commercial assays for measles IgG and varicella zoster virus IgG (Trinity Biotech, USA) were used according to the manufacturer's instructions. OD index of ≥1.1 is regarded as positive in both assays. The serum sample collected at Day-10 from this patient was positive for varicella zoster virus IgG (OD: 6.358) and measles IgG (OD: 2.553).

Cytokine Bead Array

A custom 10 cytokines LegendPlex (Biolegend, USA) panel which included CCL3 (MIP-1α), IFN-α2, IFN-γ, IL-2, IL-4, IL-5, IL-9, IL-10, IL-13, and IL-21 was used according to the manufactures instructions. Samples were acquired by flow cytometry on a FACS Attune (Invitrogen) and analyzed with LegendPlex software as per manufacturer's instructions. Samples included case 564 (day 10, 43 and 148, and negative controls (healthy donors, pre-pandemic, no current infection, n=7). Patient sera and controls were run in parallel on the same occasion.

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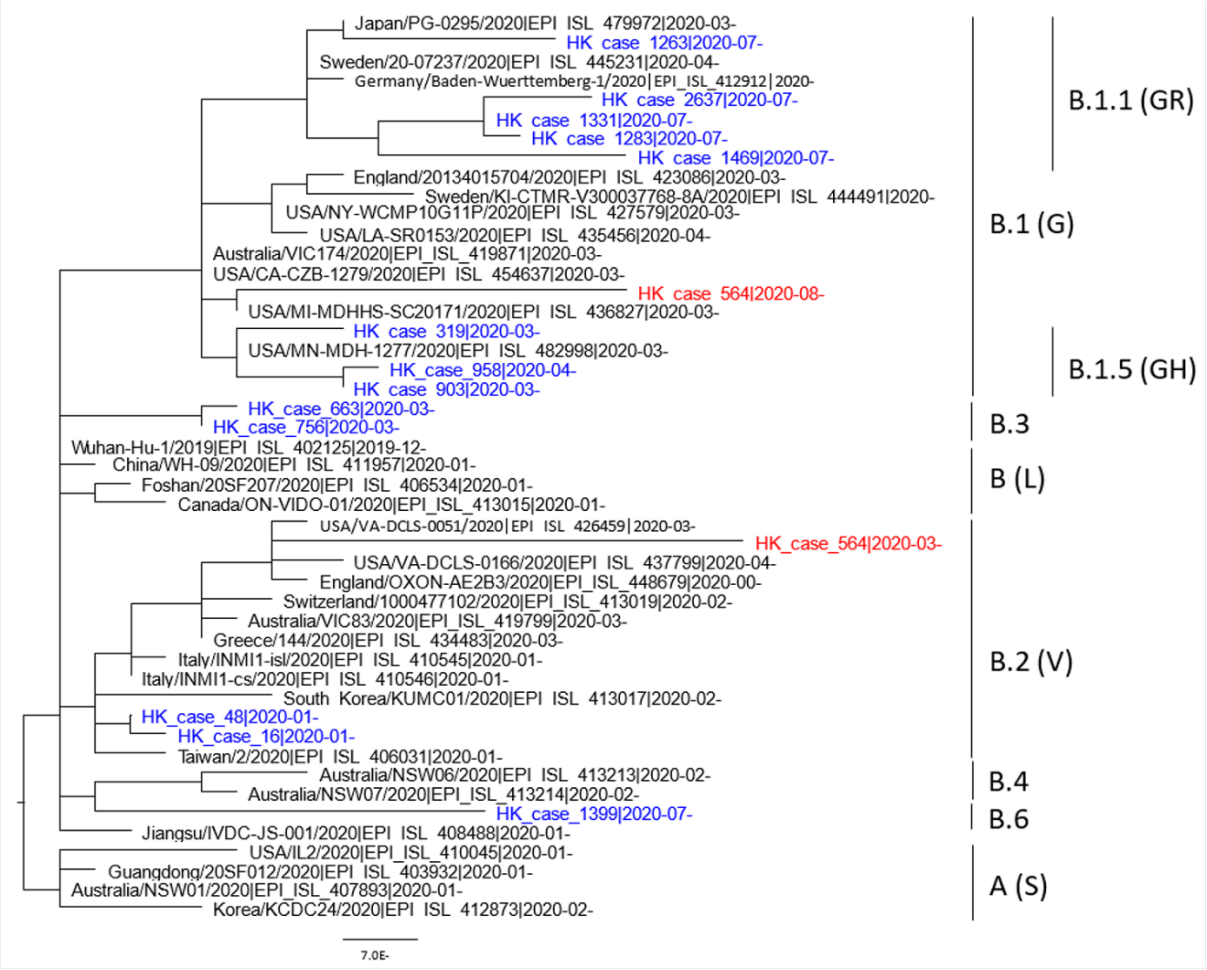
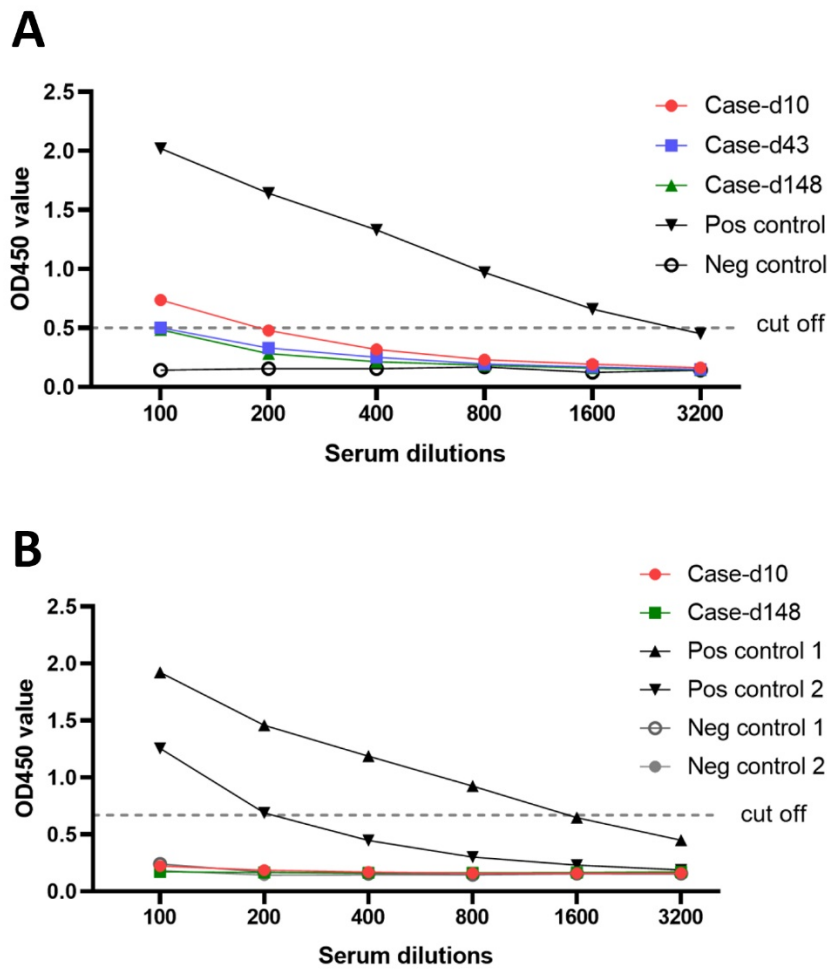
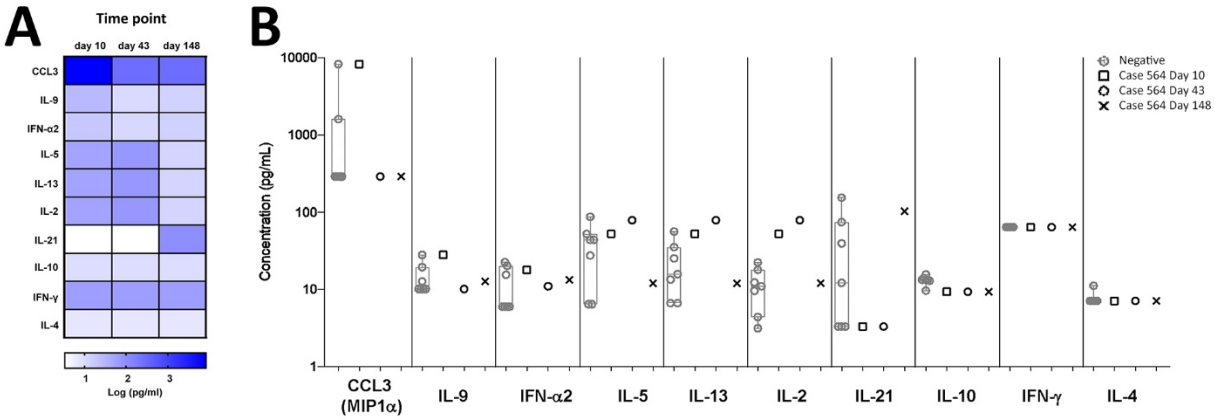


Appendix Figure 1. Phylogenetic tree of selected severe acute respiratory syndrome coronavirus 2 isolates. Red indicates viruses isolated from patient 564, Hong Kong, 2020. Patient 564 was sampled during his first infection in March and the second infection in August. Blue indicates viruses from other recent Hong Kong cases. Phylogenetic tree constructed using PhyML. Symptom onset date (YYYY-MM-DD) and GISAID access no. of the reference genomes are shown in virus taxa. Virus lineages are indicated.



Appendix Figure 2. Serum IgG and IgM responses to spike receptor binding domain in patient with selected severe acute respiratory syndrome coronavirus 2 reinfection, Hong Kong, 2020. Serial dilutions of serum samples from the patient were tested in ELISA selective for IgG (A) and IgM (B) against SARS-CoV-2 receptor binding domain. Positive and negative control serum samples included for comparison. The dotted line represents the cut-off in the previously validated assay (*1*).



Appendix Figure 3. Pro- and antiinflammatory cytokines and chemokines measured by cytokine bead array in a patient with severe acute respiratory syndrome coronavirus 2 reinfection, Hong Kong, 2020. (A) Cytokine and chemokine levels in patient on days 10, 43, and 148 after symptom onset. (B) The patient's response at different time points (black symbols) were compared to those of 7 negative controls (open gray circles). Data represent individual responses. Box plots show interquartile ranges. IFN, interferon; IL, interleukin; CCL3, chemokine (C-C motif) ligand 3; MIP-1α, macrophage inflammatory protein-1 α.