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Sequencing the SARS-CoV-2 Genome from Stool Samples of Post-acute Cases Implicates a Novel Mutation Associated with Reduced Antibody Neutralization

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ABSTRACT

Whole-genome SARS-CoV-2 sequencing tools are crucial for tracking the COVID-19 pandemic. However, current techniques require sampling of actively infectious patients following COVID-19 testing to recover enough SARS-CoV-2 RNA from the nasopharyngeal passage, which rapidly clears during the first few weeks of infection. A prospective assessment of the viral genome sourced from recovered non-infectious patients would greatly facilitate epidemiological tracking. Thus, we developed a protocol to isolate and sequence the genome of SARS-CoV-2 from stool samples of post-acute SARS-CoV-2 patients, at timepoints ranging from 10-120 days after onset of symptoms. Stool samples were collected from patients at varying timepoints post-convalescence, and viral DNA was isolated and sequenced using the QIAamp Viral RNA Mini Kit (Qiagen Inc.) and Ion Ampliseq™ Library Kit Plus (Life Technologies Corporation). Capacity of neutralizing antibodies in patient plasma was tested using a Luminex panel (Coronavirus Ig Total Human 11-Plex ProcartaPlex™ Panel, ThermoFisher). Of 64 samples obtained from post-acute patients, 21 (32.8%) yielded sufficient material for whole-genome sequencing. This allowed us to identify widely divergent phylogenetic relativity of the SARS-CoV-2 genome from post-acute patients living in the same households and infected around the same time. Additionally, we observed that individuals who recovered from infection expressed varying degrees of antibodies against SARS-CoV-2 structural proteins that corresponded to distinct variants. Interestingly, we identified a novel point mutation in the viral genome where infected patients expressed antibodies with a significantly reduced capacity to neutralize the virus *in vitro* relative to that of those infected with the wild-type strain. Altogether, we demonstrate a protocol to successfully sequence the SARS-CoV-2 genome from stool samples from patients up to 4 months post-infection, which can be applied to studies that assess the relationship between variants and immune response *post-hoc* and safe monitoring of the SARS-CoV-2 genome during the pandemic.

Keywords: COVID-19, sequencing, stool, variants.

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I. Introduction

At the end of 2019, an outbreak of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), in Wuhan, China, quickly grew into the worldwide COVID-19 pandemic [1]. As of 10 May 2022, there have been over 500 million confirmed cases of COVID-19 globally, with over 6.25 million confirmed deaths, including one million in the U.S [2]. SARS-CoV-2 is thought to be a novel recombinant virus of the Coronaviridae family and, like SARS-CoV and Middle East Respiratory Syndrome Coronavirus (MERS-CoV), has a zoonotic origin, likely bats or pangolins [3]. SARS-CoV-2 is a β-coronavirus, containing a ~29 kb positive-sense RNA genome [4], whose pathogenesis of human infection primarily affects the respiratory tract and varies in severity from mild symptoms to severe respiratory failure [5], concurrent with cough, fever, myalgia, dyspnea, headache, and gastrointestinal (GI) symptoms such as nausea and diarrhea [6].

Like SARS-CoV, SARS-CoV-2 relies on angiotensin-converting enzyme 2 (ACE2) as a receptor to invade human host cells [7]. Tissue/cell type-specific variability in ACE2 expression contributes to SARS-CoV-2 tissue tropism. For

example, Wang *et al.* showed that SARS-CoV-2 RNA is best detected in bronchoalveolar lavage fluid specimens [8], although viral RNA has also been found in sputum, nasal and pharyngeal swabs, feces, and urine [8]-[9]. Interestingly, Li *et al.* showed that while lungs express moderate levels of ACE2, higher expression may occur in the small intestine, testis, kidneys, heart, thyroid, and adipose tissue [10]. While viral RNA has been detected in post-mortem myocardial tissues, viral load did not correlate with degree of symptomatic cardiac involvement [11]. Similarly, male reproductive tissues are thought to be potential targets of SARS-CoV-2, due to high ACE2 expression in Sertoli, Leydig, and germ cells [12]–[13].

In addition to clinical studies, in vitro human organoid studies showed that SARS-CoV-2 can infect blood vessels, kidney, liver, and small and large intestines [14]–[16]. Wang *et al.* further showed that the live virus could be detected in feces, supporting the GI tract as a target organ system for SARS-CoV-2, suggesting a fecal route of transmission (8). Analogously, Lin *et al.* detected viral loads in the esophagus, stomach, duodenum, and rectum via endoscopic sampling [17], while Xiao *et al.* showed that SARS-CoV-2 can infect and enter GI cells [18]. This group also detected the virus in feces of patients, despite negative respiratory samples, suggesting that the virus can potentially persist in the GI tract longer than in the nasopharynx and the respiratory tract.

Given the rapid clearance of SARS-CoV-2 from the nasopharyngeal after the first few weeks of infection, the persistence of the virus in the GI tract and stool samples may provide an alternative means to study viral variants genetically identified post hoc in non-infectious, post-acute individuals. A recent study demonstrated the feasibility of viral detection in stool samples of patients for as many as 77 days after infection [19], though more normally stool samples have returned positive results up to 33 days after a negative nasopharyngeal test [20]. However, whether the full viral genome remains intact in stool sample and amenable to sequencing has not yet been demonstrated. Herein, we describe a protocol to isolate and sequence the genome of SARS-CoV-2 from stool samples of post-acute patients and demonstrate an example of how this data was applied to understanding the interindividual variability in the immune response to infection during convalescence.

II. Methods

A. Sample Collection

All human subject studies were approved by the Institutional Review Board of the University of Hawaii under protocol number 2020-00411. All participants provided informed consent during enrollment. In addition, informed consent for children under 18 years old was obtained from a parent and/or legal guardian. All methods were performed in accordance with the relevant guidelines and regulations. Data was collected from Oahu residents who had tested positive for COVID-19 during recruitment, but less than 3 months before the first point of data collection. Patients could only enroll in the study if they were considered noninfectious and "cured" by their physician. Males and females, aged 5 to 78 years old at the time of recruitment, were invited for weekly follow-up to the research clinic for 6 weeks, where blood samples were collected at each visit. During the 2nd week, patients also provided a stool sample and were measured for height and weight (for BMI calculation). All participants, and their respective survey responses, were anonymized with a unique numerical ID, to ensure privacy. Data from patients that missed more than two appointments were excluded from the analyses. Informed consent was provided to all patients, and assent was given before participation in the study.

B. Viral RNA Extraction from Stool Samples

To clarify stool samples, 0.5 mL stool aliquots, preserved in RNAlater™ Stabilization Solution (Thermo Fisher Scientific, Inc., Vilnius, Lithuania), were added to 2.5 mL of 0.89% NaCl solution and centrifuged for 20 min at 4000 g. The supernatant was collected and filtered through an 0.2 µm syringe filter. Then, 2 mL of the filtrate was concentrated in Amicon® Ultra-2 mL centrifugal filters at 4000g, in 10 min intervals, until ~ 140 µL remained in the column, which was then recovered by inverting the column and spinning at 1000g for 2 min. The recovered sample concentrates were processed via the QIAamp Viral RNA Mini Kit (Qiagen Inc., Valencia, CA, USA) for RNA extraction. Next, RNA (2 µL) was converted to cDNA using the SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA), following the product's directions. The final cDNA product was then diluted in RNase-Free water (1:2).

C. Library Preparation and Sequencing

The Ion Ampliseq™ Library Kit Plus (Invitrogen) was used to amplify viral RNA extracted from stool samples. Following the amplification procedures indicated in the manufacturer's protocol, PCR was performed in two pools, each including specific primer sets. After PCR amplification, full volume reaction products were run on E-Gel precast agarose gels (Invitrogen). Successfully amplified PCR products were cut from the gel and pools 1 and 2 of each sample were combined into a single 1.5 mL tube. Then, PCR products were purified using GeneJET Gel Extraction Kits (Thermo Fisher Scientific), following the manufacturer's directions. To ensure sufficient amplification, a second PCR reaction was performed with the two primer pools using Platinum™ PCR SuperMix High Fidelity (Invitrogen). After the second amplification reaction, DNA libraries were prepared according to the Ion Ampliseq™ Library Kit Plus (Invitrogen) protocol. First, the amplicons were partially digested, and adapters (Ion Xpress Barcodes Adapters 1–80 Kit, Invitrogen) were then ligated according to manufacturer's protocol. Lastly, the libraries were purified using Agencourt™ Ampure™ XP beads (Beckman Coulter, Brea, CA, USA), and their concentrations were determined by qPCR using the Ion Universal Library Quantification Kit (Invitrogen), per the kit's instructions. Then, each library was diluted to a concentration of 39 pM, and equal volumes of each library were pooled. The Ion 510™ & Ion 520™ & Ion 530™ Kit (Thermo Fisher Scientific, Austin, TX, USA) was used to clonally amplify the pooled library on nanosized ionosphere particles by emulsion PCR. Bead enrichment and chip loading were also conducted using the Ion Chef Instrument. The parallel sequencing was achieved on the Ion S5 Next-Generation Sequencing system with Ion 530 chips.

D. Luminex Panel of anti-SARS-CoV-2 Antibodies

Plasma collected from participants was evaluated against WT SARS-CoV-2 structural proteins using a Luminex panel (Coronavirus Ig Total Human 11-Plex ProcartaPlex™ Panel, ThermoFisher, Vienna, Austria), following the manufacturer's protocol. The panel was read using a Luminex 200 Instrument System (Thermo Fisher Scientific).

E. SARS-CoV-2 Surrogate Virus Neutralization Assay

SARS-CoV-2 Surrogate Virus Neutralization Test (sVNT) Kit (GenScript, NJ, USA) is a blocking ELISA which mimics the virus neutralization process, detecting circulating neutralizing SARS-CoV-2 antibodies that block the interaction between RBD and ACE2 on the cell surface receptor of the host. The test is isotype and species independent. Plasma samples were diluted 10X with sample dilution buffer and assayed following GenScript protocol. The absorbance of the sample is inversely dependent on the titer of the anti-SARS-CoV-2 neutralizing antibodies. S-RBD (wild type) was used in this assay.

F. Statistical Analysis

Comparative analyses of immunological data between different lineages were performed using a One-way ANOVA test, followed by Tukey multiple comparison test. An unpaired t-test was used to evaluate the impact of punctual mutations on inhibition assay. Graphing and statistical analysis were performed using Prism 9, Version 9.0c (GraphPad, La Jolla, CA, USA). All statistical significance was determined at $P < 0.05$.

III. Results

A. Recruitment, Sample Processing, and Sequencing

We recruited study participants during the first wave of SARS-CoV-2 infections in Honolulu, Hawaii, between June and September 2020. The individuals from whom samples were collected represented a range of ages, ethnicities, and sexes, with enrichment of Native Hawaiians and other Pacific Islanders (NHPI) that comprise approximately 25% of the state's population (Table I). Fig. 1a illustrates the 7-day average of reported cases over time in Hawaii, showing multiple surges in COVID-19 cases as a result of dominant variants, including Delta and Omicron, in relation to the Alpha/WT variant surge suspected to have been prevalent during our recruitment period.

Fig. 1b illustrates the workflow used to amplify the SARS-CoV-2 RNA from stool samples. Of 64 donor stool samples, 21 (32.8%) were successfully sequenced, showing persistence of the viral genome in stool up to four months after infection (Fig. 1c). Of the 43 samples that could not be sequenced, 5 failed the sample collection and filtration step, 35 failed to amplify after the first PCR step, and 3 were lost following sequencing due to insufficient numbers of reads.

TABLE I: Sociodemographic Characteristics of Total Study Participant Population

	Number	Percentage
N	67*	
Sex		
Female	40	60%
Male	27	40%
Age (years)		
Less than 30	19	28%
From 30 to 55	35	52%
Greater than 55	13	20%
Ethnicity		
White	21	31%
NHPI	27	44%
Asian	14	21%
Other	5	4%
Major Symptoms		
Fever	23	34%
Cough	23	34%
Muscle Pain	4	6%
Headache	18	27%
Sore Throat	30	45%
Loss of Taste or smell	16	24%
Trouble breathing	27	44%
HbA1C (% in blood)		
Healthy (Below 5.7%)	54	81%
Prediabetic (Between 5.7% and 6.4%)	0	0%
Diabetic (Over 6.5%)	8	12%
Did not want to get tested	5	7%
Blood pressure category		
Normal (systolic below 120, diastolic below 80)	22	33%
Prehypertension (systolic between 120-139, diastolic between 80-89)	31	46%
Hypertension (systolic above 140, diastolic above 90)	7	10%
Did not want to get tested	7	10%

All 21 sequenced samples exceeded quality control thresholds (described in Table II). Sequenced libraries generated from these samples had an average of 91% mapped reads, 35X mean depth, and a mean 85.6% quality score of Q20, indicating 99% base call accuracy. These data indicate high quality sequencing results. As shown in Fig. 2a, the largest absolute number of successfully sequenced SARS-CoV-2 genomes were from samples collected within a month after the onset of symptoms. However, the proportion of successfully sequenced genomes did not significantly depend on convalescence, as the highest percentage of successful sequencing was within the 81-90 day interval, with 100% of the samples sequenced (Fig. 2b). This suggests that time after infection may not be crucial for successful sequencing of the SARS-CoV-2 genome from fecal samples. The ability to amplify the samples may also depend on technical factors, such as sample storage condition and duration, or biological variables such as individual viral load and shedding.

Sequencing identified several SARS-CoV-2 subvariants present in the tested population, whose distribution is shown in Fig. 2c, showing predominance of B1.243 (27.26%) and B.1 (13.64%) subvariants, appearing earlier than reported by the state of Hawaii in May 2021 (Hawaii Department of Health, 2021). Our data was collected in June to September 2020, while the report describes data collected in the two weeks prior to the date shown, beginning in May 2021.

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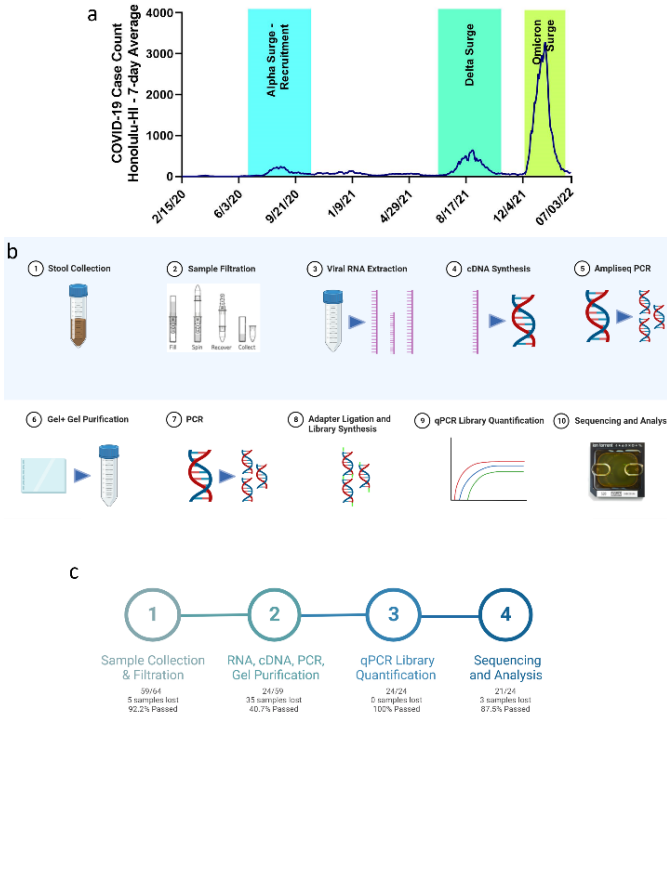

Fig. 1. Successful isolation of viral RNA and sequencing from patient stool samples. a) 7-day average case count, as reported by Hawaii Department of Health, with surges due to WT, Delta, and Omicron variants highlighted. b) Experimental design: viral RNA was extracted from filtered stool samples, converted to cDNA, and amplified by PCR. After electrophoresis e-gel isolation and purification, further PCR was performed, and 16S libraries were created by adapter ligation, quantified by qPCR, and sequenced using the Ion S5 Next-Generation Sequencing system with Ion 530 chips (Created with BioRender.com). c) Processing of samples were unsuccessful at different steps in the protocol, resulting in a total of 21/64 successfully sequenced samples (Created with BioRender.com).

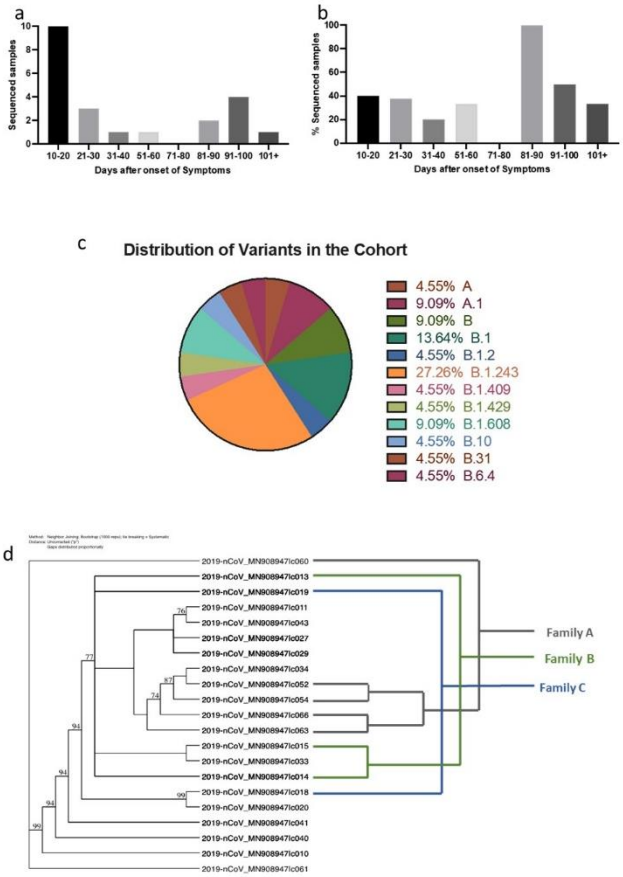

Fig. 2. Successful amplification of viral RNA is not dependent on collection time. a) Number of samples successfully sequenced from patient samples separated by time after onset of symptoms. b) Percentage of samples at each timepoint that were successfully sequenced. c) Distribution of SARS-CoV-2 variants identified in all patient samples. Samples were collected between June and September 2020 in Oahu, HI, USA. d) Phylogenetic analysis indicates a rapid rate of viral mutation. Three family groups living together (A, B, and C) were found to have largely divergent variants of the virus within their family groups.

TABLE II: QUALITY CONTROL OF NEXT GENERATION SEQUENCING

Sample	Bases	Reads	Mean Read length	Mapped Reads	Mean Depth
c009	1,198,205,462	6,324,538	189	6,044,096	36,938
c010	1,489,569,391	7,998,512	186	7,659,778	46,434
c011	1,420,405,554	7,782,617	182	7,351,799	43,158
c013	1,265,373,919	7,130,732	177	6,785,235	37,972
c014	896,894,202	5,145,390	174	5,009,620	27,482
c015	1,402,887,604	7,838,674	178	7,629,091	43,051
c020	1,094,646,127	6,366,095	171	6,134,237	33,530
c019	1,258,534,760	7,055,747	178	6,932,524	40,648
c018	1,268,627,314	6,923,648	183	6,642,191	37,989
c029	1,250,649,554	7,022,447	178	6,719,011	37,883
c027	1,833,125,595	10,128,995	180	9,407,724	52,309
c060	521,208,581	2,975,609	175	1,895,676	2,749
c063	1,045,161,100	6,184,851	168	4,970,854	29,312
c066	999,836,546	5,145,831	194	5,042,641	31,993
c061	817,345,387	4,406,087	185	2,536,814	105.4
c033	1,360,821,401	7,702,775	176	6,762,678	32,315
c034	905,566,710	4,983,346	181	3,296,075	20,372
c040	979,524,142	5,468,628	179	5,293,151	30,302
c041	1,457,526,880	7,898,404	184	7,654,353	46,282
c043	1,176,684,729	6,547,919	179	6,463,230	38,207
c052	1,529,825,053	7,889,273	193	7,848,024	50,528
c054	1,670,225,938	8,761,159	190	8,694,724	54,565
average	1,220,120,270	6,712,785	181	6,216,978	35,187
SEM	64342038.53	340133.86	1.43	400391.91	2937.02

According to the state report, 90% of all sequenced genomes at the time were subvariants B.1.429 and B.1.1.7. During our recruitment period, B.1.429 only made up 4.55% of the subvariants present, while B.1.1.7 was fully absent from the population tested. However, in the few short months between September 2020 and May 2021, the dominant variants in the population changed, and new variants emerged or predominated (Fig. 1a).

B. SARS-CoV-2 Strains in Families

To determine the relationship of viral subvariants within families, we performed phylogenetic analyses that revealed the viral genome to differ significantly within identified patient family clusters (Fig. 2d). Three patient families were identified, termed Families A, B, or C. Since individuals within each family lived together and reported concurrent symptoms, they were likely infected with SARS-CoV-2 at the same time, with the same or similar subvariant. However, genome data demonstrated sequentially divergent subvariants within each family cluster. For example, one Family A member had a subvariant with 619 nucleotide changes relative to a subvariant found in another Family A member. This may imply a rapid mutation rate or multiple concurrent subvariants in the population.

C. Antibody Neutralization of Distinct Viral Strains

Based on prior studies showing interindividual differences in immune responses against SARS-CoV-2 [21], we explored how immune response may vary based on viral genotype. We first measured antibody levels against SARS-CoV-2 from plasma of post-acute patients in our study. The levels of antibodies against the nucleocapsid, spike, spike subunit 1, and RBD structural proteins varied across individuals in association with specific SARS-CoV-2 strains (Fig. 3a-d). In contrast to the highest levels of antibodies observed against all structural proteins in patients infected with subvariants B1.608 and B.31, low to no antibodies were detected against these proteins in individuals infected with the B.10 and B.1.409 subvariants. Further, individuals recovering from infection with the B.1.409 and B.10 subvariants exhibited a lower in vitro neutralization capacity of SARS-CoV-2 than that of others (Fig. 3e). In addition to these subvariants, we identified two novel missense mutations in the gene encoding the nucleocapsid (N) protein of SARS-CoV-2. The mutation 419A>G had no effect on the capacity of antibodies from post-acute SARS-CoV-2-infected patients to neutralize the virus in vitro (data not shown).

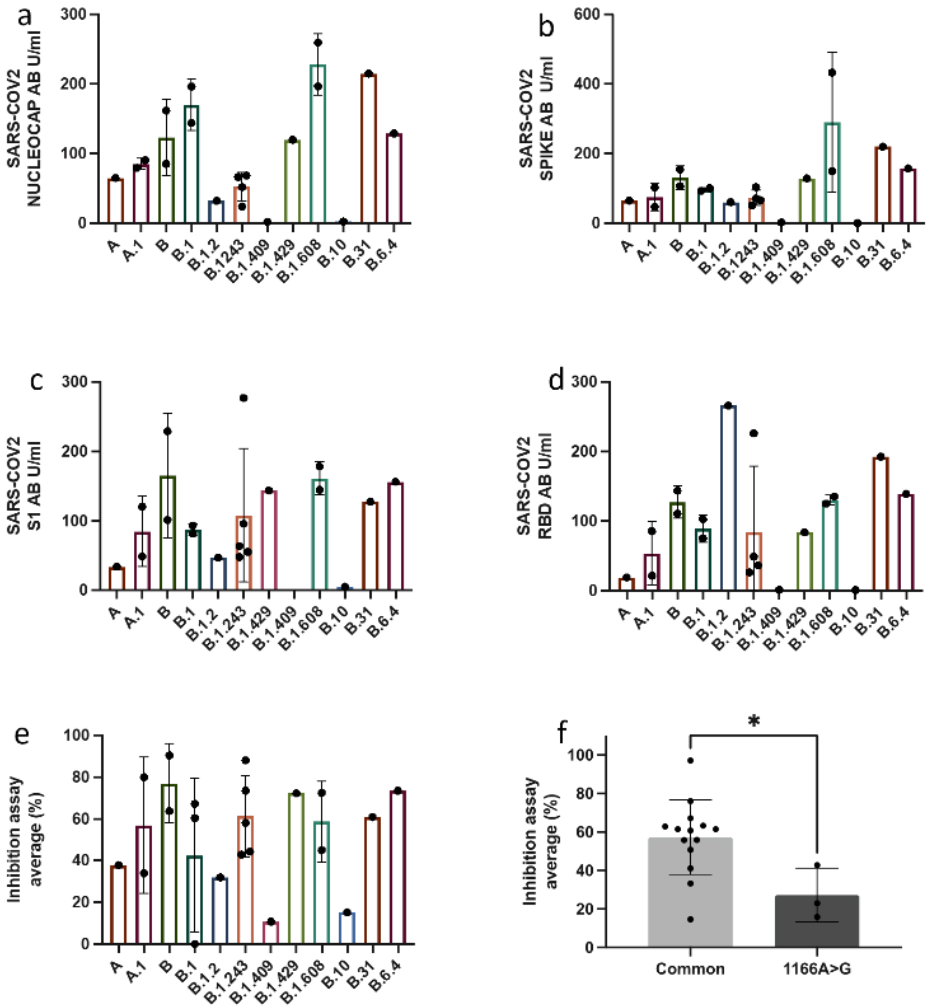

Fig. 3. A novel point mutation in our cohort reduces neutralization ability of SARS-CoV-2 in patient plasma samples. a-d) Neutralization ability was assessed using patient plasma samples against SARS-CoV2 nucleocapsid (a), spike (b), spike S1 subunit (c), and receptor-binding (d) domains, for each identified variant. Higher levels of neutralization indicated higher seroprevalence of SARS-CoV-2 protein-specific antibodies. e) Total inhibition ability against the entire virus was assessed by variant. Data shown is the average percentage of successful neutralization of each variant by patient plasma samples. f) The ability of patient plasma samples to neutralize a newly identified variant with a point mutation 1166A>G was compared to the WT variant. A significant decrease in neutralization capacity was identified in the mutant, compared to common variant (one-tailed t-test).

However, the mutation 1166A>G exhibited reduced capacity to neutralize the virus in vitro by 30% relative to post-acute COVID-19 patients without this mutation (Fig. 3f).

IV. Discussion

The results of this study demonstrate the success and utility of a protocol to isolate and sequence the genome of SARS-CoV-2 from stool samples of post-acute patients, which can enable prospective studies to better understand the heterogeneity in the sequalae of post-acute COVID-19. Although viral sequencing from fecal samples is a well-established technique in virology and has been used to characterize infections of many different viruses [22]–[25], our current study represents the first time a SARS-CoV-2 viral genome has been completely sequenced from stool samples of fully recovered patients. While previous studies have used other methods to sequence fecal samples from acute SARS-CoV-2 patients, our study uses a safer, easier, and cheaper method than previously described [26]–[28]. Additionally, we demonstrate the possibility of high-quality sequencing of the full SARS-CoV-2 genome for up to 120 days after infection. This indicates that samples may be collected without the use of a nasopharyngeal swab or the presence of active sickness or infection.

Although previous studies have demonstrated the utility of fecal swabs for early diagnosis or tracking viral circulation through wastewater [27], [29]–[30], we demonstrate a utility for individual samples for evaluation in a prospective cohort of recovering patients. This allows for better, less invasive tracking and characterization of various viral strains that emerge over the course of the pandemic. Information collected in this manner may be used to improve strategies for COVID-19 mitigation strategies such as vaccination and quarantine. Indeed, studies reveal that more breakthrough cases may not only relate to waning vaccine-induced immunity, but also associate with specific viral variants [31]–[33], indicating a clear need to continue monitoring changes in the distribution of variants over time and assess whether boosters, vaccines, or anti-viral drugs may be warranted.

As an exploratory application of our method, we further examined the novel mutations we identified and the way they might affect the activity of the virus. Prior studies show substantial interindividual variation in immune response against SARS-CoV-2 [21]. Although individual innate features such as genetics, gut microbiome, age, sex, and obesity may contribute to this variability [34]–[38], it is unclear whether distinct mutations or viral subvariants also account for varying individual immune responses. We observed that individuals infected with distinct subvariants exhibited varying degrees of anti-SARS-CoV-2 antibody production. In particular, we found that individuals recovering from SARS-CoV-2 carrying a novel mutation (1166A>G) in the nucleocapsid (N) protein had significantly reduced viral neutralization capacity in vitro. We caution that these results are preliminary, as the cohort size was small, with asynchronous recovery periods from infection, altogether limiting our assessment of covariates (e.g., age) that may also contribute to interindividual differences in the immune response. However, these results support that genetic variation, intrinsic to SARS-CoV-2, may at least in part explain the interindividual variability in immune responses observed, warranting further investigation. These findings demonstrate the utility and relevance of our method in the context of a rapidly changing pandemic.

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Conflict of Interest

Authors declare that they have no conflicts of interest.

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