SARS-CoV-2 Viral RNA Load is Associated with the Number of COVID-19 Symptoms Experienced in Mildly Symptomatic Adults: An Observational Study

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INTRODUCTION

COVID-19 is associated with a myriad of clinical manifestations ranging from none (asymptomatic) to critically ill patients [1], affecting multiple systems. Recent meta-analysis studies estimate that 20-35% of SARS-CoV-2 carriers are asymptomatic [2-4]. As part of Celerion's COVID-19 mitigation strategy, early on in the pandemic we established ultrasensitive SARS-CoV-2 PCR assays to test and track asymptomatic and symptomatic associates and study participants within our pharmacology clinics [5]. With a significant proportion of COVID-19 infections deriving from asymptomatic or mildly symptomatic patients, little has been documented for these groups, especially with respect to the time course of viral infection and antibody response. Therefore, the objectives of this study were to assess SARS-CoV-2 nasopharyngeal and saliva viral kinetics as well as antibody titers from asymptomatic and mildly symptomatic individuals since first positive COVID-19 PCR test result until infection was no longer detectable.

METHODS

Study Design and Participants

As part of risk mitigation procedures, routine COVID-19 screening was performed on all employees, contractors, study participants and visitors entering Celerion's clinical research unit (CRU). Briefly, nasopharyngeal samples were collected and screened for the detection of SARS-CoV-2 RNA by PCR (as described below). Anyone detected positive for the virus at the Tempe, AZ CRU between September 2020 and April 2021, was invited to take part in an observational study. Eligible participants must have tested positive within 14 days of enrollment and were required to have previously tested negative using Celerion's PCR assay. Subjects were excluded if: they were currently taking antiviral medication; had participated in COVID-19 vaccine or antibody therapies clinical trial; or had participated in a clinical trial involving immune system modulators or anti-viral drugs within the past 3 months. The screening procedure and observational study were reviewed and approved by the Advarra Institutional Review Board (Columbia, MD), and all participants provided signed informed consent.

Study participants returned to the CRU's designated COVID-19 Screening Area every 2-3 days for the first three weeks (Study Days 1 to ~19) for paired nasal swab, saliva and blood collection, and returned on Study Day 28, 42, and 56 for blood collection. Participants were queried about symptoms at each visit. Participants meeting a symptoms threshold were asked to refrain from visiting the CRU until their symptoms resolved, instead a telephone call visit was conducted for the health and safety of the participant as well as a precaution to minimize associate interaction with actively symptomatic COVID-19 carriers. The symptomatic threshold included active symptoms of fever (body temperature ≥ 100°F), chills, shortness of breath or difficulty breathing, persistent cough, or new loss of taste and smell; or two or more of the following: fatigue, muscle or body aches, headache, sore throat, congestion or runny nose, nausea or vomiting, diarrhea, hair loss, brain fog or other symptoms.

Twenty-nine participants completed the study. One subject discontinued at visit 7 due to personal reasons. Six participants reported receiving one dose of the COVID-19 vaccine (Pfizer-BioNTech) during the observational trial period, and one participant received two doses of the vaccine. Vaccinated participants were further queried regarding vaccine-related symptoms such as injection site pain, swelling and rash. Vaccine-related symptoms were not considered in the analysis.

Nasopharyngeal and Saliva Quantitative PCR Assay Development and Validation

We established and validated an extraction-free ultrasensitive real-time polymerase chain reaction (qPCR) assay using CDC recommended and primers/probe sets as described in a separate report [5]. Briefly, nasopharyngeal samples were collected in viral transport medium (Hardy Diagnostics, Santa Maria, CA) and plated onto a 96-well optical plate (Applied Biosystems, Waltham, MA). Samples were heat inactivated and lysed for 5 min at 98°C in a thermal cycler (SimpliAmp Thermal Cycler, Applied Biosystems). Primer/probe sets for 2019-nCoV selected from the nucleocapsid gene (N1) and human RNase P gene (RP) (Integrated DNA Technologies, Coralville, IA), along with reaction mix (Reliance One-Step Multiplex RT-qPCR Supermix, BioRad, Hercules, CA) were added to the plate and loaded into the detector (7500 Real-Time PCR System, Applied Biosystems) for transcription and amplification. A standard curve was prepared using Heat-Inactivated 2019-nCoV (Integrated DNA Technologies) and samples were reported as genome copies/uL. The limit of detection was 0.5 genome copies/uL [5]. Screening (first positive COVID-19 test) PCR Ct values were extrapolated to genome copies/uL from an average standard curve.

Approximately 2mL of saliva was collected in a Saliva RNA Collection and Preservation System (Somru BioScience, Canada). Total nucleic acids were isolated with Maxwell® RSC Viral Total Nucleic Acid Purification Kit (Promega, Madison, WI). Transcription and amplification methods were performed as described above. The Heat-Inactivated 2019-nCoV standard curve and control samples were treated under the same conditions as the saliva samples, and results are reported as genome copies/uL. The limit of detection was 1.0 genome copies/uL [5].

Antibody Assay

Serum samples were collected approximately on Study Days 1, 7, 13, 19, 28, 42, 56 to measure the immune response mounted using antibody assays against the SARS-CoV-2 spike protein (COBAS Elecsys Anti-SARS-CoV-2 S, Roche, Switzerland). Assay level of detection ranged from 0.4 to 2500 U/mL.

Statistical Analysis

Results are presented as mean ± standard deviation (SD). Log-transformed viral RNA and antibody data was analyzed using nonlinear regression with a one-phase decay or association model respectively. Separate nonlinear regression models were fit for symptomatic and asymptomatic participants and statistically compared using an F test. A p-value <0.05 suggested that separate curves provided a better fit than one curve for all data. Kaplan-Meier plots were created to visualize time to not detected or time to symptom resolution as indicated. Nasopharyngeal and saliva results were compared with a contingency analysis. Statistical analysis and graphical representations were performed with Prism, GraphPad (San Diego, CA).

RESULTS

Participant Characteristics and Symptoms

Thirty participants (11 males / 19 females) enrolled in the study. The time from first positive test to enrollment (Visit 1) ranged from 1 to 28 days (Table 1). Average age was 30 years and the majority of participants were White Hispanic or Latino. Overall participants were classified as overweight with an average BMI of 27.2±4.8 kg/m^2 yet otherwise healthy, (10% reported a preexisting medical condition of asthma, 3% hyperthyroidism, 3% hypertension). In addition, only two participants were smokers.

Table 1. Summary of Demographic Characteristics

Demographic Characteristic	Study Population (n=30)
Age (years)	30.8±8.77 (20 - 49)
Gender (Male/Female)	11 / 19
Self-Reported Race (%)	
• Asian	2 (7%)
• Black or African American	1 (3%)
• White	26 (87%)
• Not reported	1 (3%)
Self-Reported Ethnicity (%)	
• Filipino	1 (3%)
• Hispanic or Latino	17 (57%)
• Non-Hispanic or Non-Latino	10 (33%)
• Not reported	2 (7%)
Weight (kg)	166.1±38.8 (105 - 246)
BMI (kg/m^2)	27.2±4.8 (18.6 – 36.3)
History of Smoking	
• Non-smoker	28 (93%)
• Smoker	2 (7%)
Medical Conditions	
• Asthma	3 (10%)
• Hyperthyroidism	1 (3%)
• Hypertension	1 (3%)
• Heart murmur	1 (3%)
• GERD	1 (3%)
• Suspected lupus	1 (3%)
Number of Days from 1st Positive Test to Visit 1	9.8±7.1 (1 - 28)

Data presented as mean±SD with range in parenthesis or value and percentage.

Six participants remained asymptomatic throughout the entire study. Of the participants that were symptomatic, the majority only reported 1-2 mild symptoms since first testing positive and/or throughout the course of the study. The most commonly reported symptoms include headache, fatigue, cough, congestion or runny nose, and loss of taste and/or smell (Table 2). All symptoms were mild in nature and no subject required hospitalization for their symptoms.

Table 2. Summary of Symptoms

COVID-19 Symptoms	Number of Participants (%)
Total Number of Subjects	30 (100%)
Number of Asymptomatic Subjects	6 (20%)
Number of Subjects reporting 1-2 Symptoms	11 (37%)
Number of Subjects reporting 3-5 Symptoms	9 (30%)
Number of Subjects reporting >5 Symptoms	4 (13%)
Symptoms:	
Fever (Pyrexia)	2 (7%)
Chills	6 (20%)
Persistent Cough (Tussis)	7 (23%)
New Loss of Taste or Smell (Aguesia or Anosmia)	7 (23%)
Shortness of Breath or Difficulty Breathing (Dyspnea)	4 (13%)
Fatigue	9 (30%)
Muscle or Body Aches (Myalgia)	4 (13%)
Headache	11 (37%)
Sore Throat (Pharyngitis)	3 (10%)
Congestion or Runny Nose (Rhinorrhea or Rhinitis)	17 (57%)
Nausea or Vomiting (Emesis)	6 (20%)
Diarrhea (Gastroenteritis)	4 (13%)
Newly Identified COVID-19 Symptoms: Brain Fog (Cognitive Impairment) Hair Loss (Alopecia)	 2 (7%) 2 (7%)

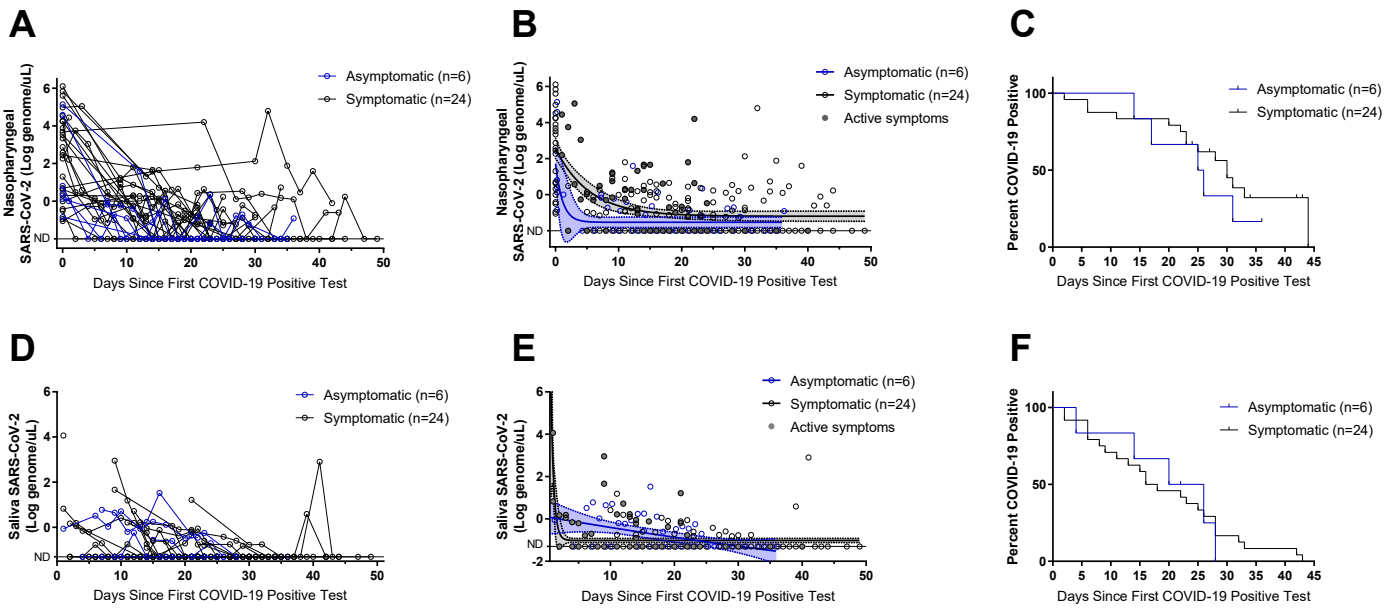
Asymptomatic SARS-CoV-2 Carriers have Lower Nasopharyngeal Viral RNA

On average, asymptomatic participants started with an initial screening SARS-CoV-2 viral load 3.6x lower than symptomatic participants and dramatically decreased viral levels 5-10 days post a first positive test (Figure 1A). While, the symptomatic group also demonstrated a drop in viral RNA by 7-fold from screening to one-week post, this was not nearly the same magnitude change seen with the asymptomatic cohort (Figure 1A). Interestingly, two symptomatic participants maintained high viral levels >20 days since their first positive test. Most symptomatic participants reported having active symptoms during the first 20 days since testing positive; and the presence of active symptoms during a study visit did not seem to result in a higher viral load (Figure 1B). Nonlinear regression modeling revealed that asymptomatic viral levels are significantly lower than symptomatic counterparts, especially over the first week since testing positive ($p=0.004$, Figure 1B). Moreover, asymptomatic participants also seem to clear the virus faster. A Kaplan-Meier plot demonstrates a slight reduction in number of days to achieving not detected status (Figure 1C).

Faster Saliva RNA Clearance Rates in Asymptomatic vs Symptomatic Subjects

From the 251 paired longitudinal samples, fewer saliva samples were detected positive for SARS-CoV-2 compared to nasopharyngeal samples (26% vs 45%), indicating a difference in detection between the two types of specimens. As such, the sensitivity and specificity (and 95% CI) for saliva compared to nasopharyngeal were 0.372 (0.288 to 0.465) and 0.826 (0.754 to 0.880) respectively. In addition, mean viral concentration was 9.7x lower in saliva compared to nasopharyngeal samples. Nonetheless, the saliva viral RNA time course for asymptomatic and symptomatic participants followed a similar trend as the nasopharyngeal samples (Figure 1D). Specifically, nonlinear regression modeling shows that initial saliva RNA levels were higher in symptomatic participants ($p<0.0001$, Figure 1E) and virus clearance was delayed (Figure 1F) compared to asymptomatic counterparts.

Figure 1. SARS-CoV-2 Nasopharyngeal Swab and Saliva Results

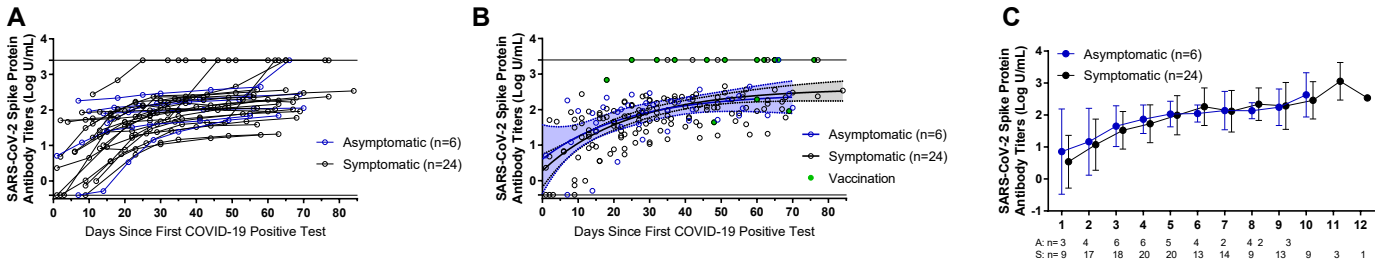


(A and B) Nasopharyngeal and (D and E) saliva SARS-CoV-2 viral level time course for individual asymptomatic (blue) and symptomatic (black) participants. Not detected (ND) results hypothetically set at -2.0 and -1.3 Log genome/µL for nasopharyngeal and saliva, respectively. (B and E) Nonlinear regression one phase decay models of asymptomatic and symptomatic, including participants with active symptoms during study visit (filled circles). Solid curves are best-fit models and shaded area represents 95% confidence intervals (CI). (C and F) Kaplan-Meier plot of days to not detected for asymptomatic and symptomatic participants. Date of first ND results followed by subsequent ND findings indicated by 1, otherwise date of a positive test results was indicated by 0.

Asymptomatic and Symptomatic Participants Display Similar Immune Response to SARS-CoV-2 Spike Protein

The time course of antibody titers demonstrated that all participants mounted an immune response to SARS-CoV-2 spike protein (Figure 2A). In general, antibody titers increased over the first 10-15 days post testing positive for SARS-CoV-2, then plateaued for the remainder of the study. Both groups demonstrate similar titer levels over the course of the study. Nonlinear regression modeling and weekly mean antibody titer display nearly superimposable lines for asymptomatic and symptomatic participants (Figures 2B and 2C). The presence of active symptoms did not have an effect on immune response in our cohort.

Figure 2. SARS-CoV2 Antibody Titers

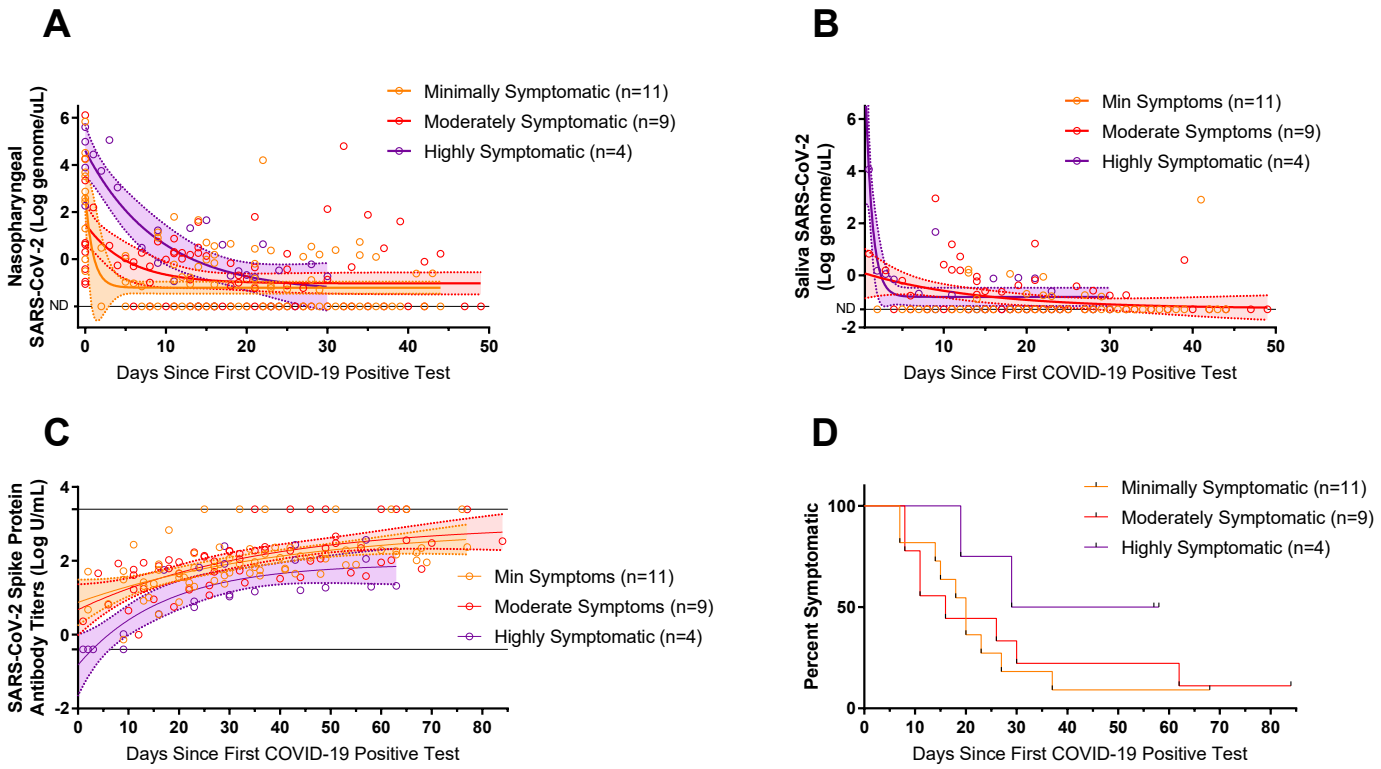


(A) Individual asymptomatic (blue) and symptomatic (black) participant SARS-CoV2 antibody titer time course. (B) Nonlinear regression one phase association models of asymptomatic (blue circles) and symptomatic (black circles). Vaccinated participants shown in green. Solid curves are best-fit models and shaded area represents 95% CI. (C) Mean weekly antibody titer for asymptomatic (A; blue) and symptomatic (S; black) subjects. Upper and lower limits of detection are depicted with a solid line.

Highly Symptomatic Participants Display Elevated Viral RNA and Reduced Immune Response

A small group of participants were considered to be highly symptomatic and reported >5 symptoms over the course of the study (Table 2). These included fever, chills, congestion, shortness of breath, fatigue, loss of taste or smell, and gastrointestinal symptoms. We defined moderately symptomatic participants as those that experienced 3-5 symptoms while minimally symptomatic participants reported the occurrence of 1-2 symptoms. Overall, highly symptomatic participants displayed elevated nasopharyngeal viral levels over the course of the study and a symptom-dependent 'dose response' emerged from the modeled data ($p<0.0001$, Figure 3A). A similar trend was observed for the saliva data, where highly symptomatic participants show elevated viral RNA ($p=NS$, Figure 3B) and the rate of detected samples over three weeks of collection was greater for this group compared to participants with moderate and minimal symptoms (Highly: 48%; Moderate: 31%; Minimal: 12%). Moreover, highly symptomatic participants also demonstrated a reduced immune response as antibody titers were lower over the course of the study compared to moderately and minimally symptomatic counterparts ($p=0.0002$, Figure 3C). Finally, while the majority of participants reported symptom resolution by their last visit, two of the four highly symptomatic participants continued to report symptoms (Figure 3D).

Figure 3. Symptom-Dependent Viral Load and Antibody Titers Response



Nonlinear regression models of minimal symptomatic (orange), moderately symptomatic (red) and highly symptomatic (purple) participants. Solid curves are best-fit models and shaded area represents 95% CI. (A) Nasopharyngeal samples analyzed by one phase decay model. ND results hypothetically set at -2.0 Log genome copies/µL. (B) Saliva samples analyzed by one phase decay model. ND results hypothetically set at -1.3 Log genome copies/µL. (C) Antibody titer samples analyzed by one phase association models. Upper and lower limits of detection are depicted with a solid line. (D) Kaplan-Meier plot of time to symptom resolution. Date when last reported symptom was resolved is indicated by 1, otherwise unresolved symptoms or date of last symptomatic visit indicated by 0.

DISCUSSION

Since the start of the pandemic, clinicians, researchers and epidemiologists have primarily focused on the symptomology and viral kinetics of SARS-CoV-2 in hospitalized patients. To better understand the viral time course in asymptomatic and mildly symptomatic subjects, we conducted an observational study in COVID-19 positive but otherwise healthy adults to track the virus until it was no longer detectable in nasopharyngeal and saliva samples. To achieve this we established and validated ultrasensitivity PCR SARS-CoV-2 assays for nasopharyngeal and saliva samples, reaching limits of detection of 0.5 and 1.0 genome copies/uL, respectively [5]. Overall, we observed quantifiable differences in SARS-CoV-2 viral RNA levels between asymptomatic and symptomatic subjects in both nasopharyngeal and saliva specimens, as well as detectable differences among mildly symptomatic subjects, with participants reporting >5 symptoms having higher viral levels and lower antibody titer than subjects with minimal or moderate number of symptoms.

Early studies found the incidence of asymptomatic SARS-CoV-2 confirmed infection ranged from 2-57% [6]. Now over two years into the pandemic this number had been further refined, a recent large meta-analysis of more than 350 studies estimates pooled asymptomaticity is 35% [4]. While our incidence of asymptomatic carriers is less than the estimated pooled percent, it is worthy to note that we did follow our subjects for 8 weeks, querying for symptoms at each visit. This allowed us to capture information on both post-acute as well as common chronic COVID-19 symptoms such as shortness of breath, fatigue and brain fog, which have been identified as part of “long-COVID” in non-hospitalized patients [7].

While not all studies agree [8, 9], emerging data demonstrate asymptomatic carriers tend to have lower viral load than symptomatic subjects [10-14], supporting our findings. Kawasuji et al. reported significantly higher mean viral load at initial nasopharyngeal sample collection in symptomatic patients compared to asymptomatic carriers [10]. Moreover, asymptomatic patients displayed higher Ct (lower viral load) compared to symptomatic patients 7-13 days after admission to the hospital [12]. In addition, severely ill patients demonstrated lower mean Ct values by 2.8 and 2.5 points in nasal and throat swab respectively, compared to mild-to-moderate cases [8], suggesting an association between viral load and degree of symptoms. Indeed, we observed a trend in increasing viral load and number of symptoms experienced in mildly symptomatic participants. We next explored whether higher viral levels would lead to slower viral RNA clearance.

On average, SARS-CoV-2 viral load or residual RNA is no longer detected 20-30 days post exposure [15-17]. Data modeling from throat swab samples found that viral load decreases towards the detection threshold (>40 Ct) at about day 21 after symptom onset [15]. Similarly, in a study of 26 asymptomatic participants, the median period from diagnosis to negative test was 21.5 days (10-36 days) [16]. Using our ultrasensitive nasopharyngeal assay, we were able to detect virus levels out to day 49, albeit the majority of participants (58% of the entire cohort) were considered not detected by day 30 since a positive COVID-19 diagnosis. Commonly, once an individual achieves one or in some cases two consecutive not detected results, they are considered virus free. It is important to note that we used a more stringent criterion to declare a participant “not detected” as we were repeatedly collecting samples over a 3-week period. The majority of participants displayed detectable levels of SARS-CoV-2 RNA in between “not detected” test days. Since we could not confirm this was residual or viral fragments, we counted these days towards a “positive” outcome. In agreement with our findings, faster viral clearance in asymptomatic individuals was observed compared to symptomatic counterparts [18]. When examining the nasopharyngeal and saliva data from our asymptomatic and symptomatic group, in both cases, the asymptomatic group tended to clear SARS-CoV-2 RNA faster, therefore suggesting the presence of symptoms was a factor associated with delayed viral clearance.

As anticipated, all study participants mounted an antibody response against the SARS-CoV-2 spike (S) protein. Despite differences in viral RNA, modelling data showed a similar response between asymptomatic and symptomatic groups, which plateaued ~15 days from first COVID-19 positive test and was maintained out to 10 weeks or more. In line with our results, Lei et al. demonstrated SARS-CoV-2 protein specific, S1 and N, IgM and IgG responses peaked 17-25 days after first exposure in a cohort of asymptomatic subjects [19]. However, only N-specific IgG could be detected after 2 months since first exposure, with S1-specific IgM and IgG trending down 40 days post exposure. Furthermore, they observed differing dynamic response in mild patients. In mildly symptomatic patients, S1- and N-specific IgG response were persistently high over 65 days [19], consistent with our findings. Discrepancies in asymptomatic response between the two studies may be

related to the detection method. We used an electrochemiluminescence immunoassay that captures predominately S-specific IgG, but also IgA and IgM, meanwhile the Lei study assessed individual antibody response via serum proteome microarray analysis. When comparing severely and mildly ill patients, Wang and colleagues detected N-IgG in both groups 9 days after onset of symptoms, and titers remained at a similar high level throughout the study [20]. On the other hand, in an analysis comparing intensive care unit (ICU) and non-ICU symptomatic patients, Sun et al. found significantly lower S-IgG response in ICU patients compared to non-ICU three weeks post symptom onset [21]. Interestingly, this association between symptom severity and reduced antibody titers seems to be S-IgG dependent, as N-IgM, N-IgG and S-IgM were all higher in ICU patients in the second and third week after symptom onset. This led the authors to conclude that reduced S-IgG response may have contributed to longer hospital stay and delayed viral clearance in ICU patients [21]. This rationale may also apply to our mildly symptomatic group, as we observed lower spike antibody titers in highly symptomatic subjects.

A limitation of our study is that we were not able to confirm infectivity or virus viability by cell culture experiments nor viral strain, and therefore rely on other studies when we suggest residual viral RNA rather than viral load was detected up to 50 days since first positive. For example, early findings from Bullard and colleagues suggested that replication-competent virus is no longer viable past 8 days of symptom onset from nasopharyngeal and endotracheal samples acquired during routine screening [22]. Therefore, despite detecting positive for SARS-CoV-2 weeks past first positive test, the likelihood of being infectious is extremely low. Another limitation of our study is related to the infection time course. Since we relied on two-week screening data and did not collect contract-tracing information, we were unable to confirm dates of exposure, and may have under- or over-estimated viral load differences depending on exposure timing. We did attempt to minimize the time between exposure and enrollment by requiring a prior negative COVID-19 test as part of inclusion criteria to limit this interval to a maximum of two weeks. Finally, it is important to note that the data collected for the highly symptomatic group is not as robust since our protocol stipulated that any participant exceeding a symptom threshold were not permitted to attend in-person visits. Nonetheless, over the course of the 8-week study we were able to collect 28 data points from 4 highly symptomatic subjects.

CONCLUSIONS

Overall, we observed a symptom-dependent relationship with SARS-CoV-2 RNA levels in nasopharyngeal and saliva samples. Our findings suggest that a viral load threshold may be a contributing factor to developing symptoms. Even among the vaccinated, suppressed viral load is associated with reduced symptoms experienced in breakthrough cases [23, 24]. Therefore, this relationship between viral load and symptoms warrants further exploration, especially as more virulent strains emerge. Importantly, Celerion used the results of this study to help inform on appropriate risk mitigation policies that ensured the safety of participants, employees, and visitors to its research facilities as the pandemic progressed.

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