

Serology profile and effects of influenza vaccination on COVID-19-positive symptomatic and asymptomatic patients

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Abstract

The COVID-19 pandemic has affected more than 6.6 million people worldwide. As the population returns to work, it is critical to develop tests that can reliably detect SARS-CoV-2-specific antibodies. Here, we present the results of a novel multiplex serology test to assess the immune response to COVID-19 in an outpatient cohort consisting of adults and children in Colorado. The IgG response was more robust in positive/symptomatic participants than in positive/asymptomatic participants. The IgM response in symptomatic participants was transient and largely fell below the detection limit 30 days after symptom onset. Influenza vaccination gave rise to milder symptomology, but did not protect against contagion. These results provide novel insight into serology profiling and the immune response to COVID-19.

Introduction

COVID-19 is an infectious disease caused by the novel coronavirus SARS-CoV-2-19. Since December of 2019, COVID-19 has spread worldwide causing over 460,000 deaths as of mid-June of 2020 (<https://www.who.int/emergencies/diseases/novel-coronavirus-2019/situation-reports>, see also^{2,4,5}). Novel COVID-19-infected pneumonia (NCIP) is characterized by fever, fatigue, dry cough, and dyspnea³. However, further study is required to accurately identify NCIP symptomology as a variety of other symptoms have been reported including vomiting, diarrhea, loss of smell and loss of taste^{1,6,7}. There is little information on symptom severity and presentation in COVID-19-positive patients and even less information on the antibody profile of asymptomatic patients infected with SARS-CoV-2. Most studies published since the first antibody tests were granted Emergency Use Authorization have focused on the detection of SARS-CoV-2 specific Immunoglobulin-M (IgM) and Immunoglobulin-G (IgG) antibodies against only one or two antigens (spike protein (SP) and/or nucleocapsid (NP))⁸⁻¹², and far fewer have analyzed the Immunoglobulin-A (IgA) antibody response in SARS-CoV-2 infected patients¹³⁻¹⁵. Therefore, additional research is needed to fully characterize the antibody response in asymptomatic and symptomatic SARS-CoV-2 infected patients.

Understanding the specific antibody profile and immune response in COVID-19 patients is critical for characterizing the progression of the disease, identifying patients with mild-moderate illness who may be asymptomatic or who have delayed symptom onset, and predicting potential long-term immunity¹⁶. COVID-19-positive patients have undetectable antibody levels in the early stages of infection^{17,18}. Liu et al. (2020) and others found that IgM antibodies were detected 4 days after symptom onset in COVID-19-positive patients and declined to undetectable levels after 4 weeks^{16,18}. IgG antibodies reached detectable levels 7 days after infection, peaked around 25 days, and remained highly elevated after 4 weeks¹⁸. Another recent study of 173 COVID-19-positive patients showed that the median seroconversion times for IgM and IgG were 11 days and 14 days post-infection, respectively¹⁷. IgM levels began to decline after 5 weeks and disappeared entirely after 7 weeks. IgG was highly elevated 7 weeks post-infection¹⁷. Given their long half-lives, IgG antibodies may remain above detectable thresholds for months or years after infection^{19,20}. While some longitudinal studies have characterized the antibody response of COVID-19 patients and health-care workers¹³, it is still unknown how long IgG may remain

elevated after symptoms have subsided and what implications sustained IgG levels have on long-term immunological memory. Virus-specific IgM and IgA antibodies for SARS-CoV-1, which shares much of its genome with SARS-CoV-2, are similarly elevated in the serum of infected patients 6-8 days past symptom onset and begin to decline after 3-4 weeks^{22,23}. Importantly, IgG titers in SARS-CoV-1 infected patients are elevated 8 weeks post-infection and remain elevated for up to 2 years which may indicate the presence of long-term immunity²⁴.

The most prominent immunoassays (IAs) are automated chemiluminescent IA (CLIA), manual ELISA, and rapid lateral flow IA (LFIA), which detect IgM and IgG produced in response to SARS-CoV-2 infection^{8,25}. In the current manuscript, we describe a novel CLIA, developed by Vibrant America Clinical Labs, that is approved by the Federal Drug Administration (FDA) for Emergency Use Authorization and can sensitively detect antibody titers in SARS-CoV-2 infected patients. This serological protein microarray technology has been validated and tested in more than 7,700 patient serum samples, and shown to detect a plurality of antibody responses including 12 antibody/antigen combinations: IgM, IgA, and IgG, against the spike 1 SP (S1 SP), receptor binding domain (RBD), spike 2 SP (S2 SP), and NP of SARS-CoV-2. In this previous study, investigators found a clinical sensitivity and specificity of 98.1% and 98.6%, respectively²⁶.

Colorado has had over 28,822 cases of COVID-19, with 1,595 deaths attributed to COVID-19, and 4,419 hospitalizations to date. Approximately 190,700 individuals in the community have undergone testing (see <https://covid19.colorado.gov/data/case-data> for updates). Colorado is ranked number 20 in the country regarding the number of cases and number 16 in the country in terms of the number of reported deaths caused by COVID-19.

In the current study, otherwise healthy, community-dwelling participants reported a variety of COVID-like symptoms to an outpatient clinic in the Denver area. None of the participants exhibited severe COVID-19 symptoms requiring hospitalization. We analyzed symptomatology and antibody profiles of participants suspected of having COVID-19 using the Vibrant novel multiplex serology test that detects IgA, IgG, and IgM antibodies against four antigens including the S1 SP, RBD, S2 SP, and NP. The aims of the current study were to identify elevated levels of IgA, IgG, and IgM antibodies in our cohort, characterize participant symptomatology in relation to the antibody/antigen positivity, and to determine differences between asymptomatic and symptomatic participants with positive serology profiles. Given our access to a variety of clinical data for this cohort, we also investigated potential protective effects of the tuberculosis vaccine bacillus Calmette-Guérin (BCG) and the influenza vaccine. Recent immunological research has suggested that these vaccinations may nonspecifically protect against other infections due to a process termed “trained immunity” which involves epigenetic changes in innate immune function that render the body better able to respond to subsequent immune challenges^{27,28}.

Results

Participant population

Serum samples from 108 participants (53 males and 55 females) between the ages of 12 and 78 (mean=42 years) living in Denver, Colorado and the surrounding suburbs, were tested for the presence of IgA, IgG, and IgM antibodies against SARS-CoV-2 antigens, including S1, RBD, S2, and NP using a multiplex chemiluminescence immunoassay²⁶. Thirty-one participants tested positive for at least one SARS-CoV-2 antibody/antigen combination (antibody titers ≥ 1.0). Seventy-six participants tested negative for all antibody/antigen combinations (titers < 1.0). Sixty-five of the participants (28 COVID-19-positive and 36 COVID-19-negative; one that did not have serology tested) completed extensive health questionnaires to identify symptom severity and potential comorbidities. Twenty-one COVID-19-positive participants and 18 COVID-19-negative participants reported experiencing one or more symptoms associated with COVID-19. As assessed by patient responses to questionnaire items related to medical history, physical activity levels, and general lifestyle practices, this population was considered to be healthy and active. This population had an average body mass index (BMI) of 25, only 21 participants reported chronic illnesses, and only 10 participants had a history of smoking tobacco or marijuana (**Table 1**). There were no significant differences in age, sex, BMI, smoking history, or the presence of chronic illnesses between the COVID-19-positive and COVID-19-negative participants (**Table 1**).

Antibody titers against SARS-CoV-2 antigens

We first confirmed that all combinations of antibody titers against SARS-CoV-2 antigens were significantly elevated in COVID-19-positive participants (n=31) versus COVID-19-negative participants (n=76) using two-tailed Independent Samples T-Tests. Titers for all 12 antibody/antigen combinations were significantly increased in COVID-19-positive versus COVID-19-negative participants (**Table 2**). Titers were not significantly different between males and females (data not shown). The Vibrant assay is unique in its ability to detect a variety of antibody/antigen combinations²⁶. Many of the other tests FDA approved for Emergency Use Authorization may be limited in their detection of COVID-19-positive participants because they only test one antibody against one antigen. Therefore, several of these other assays may incorrectly categorize COVID-19-positive patients as COVID-19-negative if that patient is not positive for the specific antibody/antigen combination used in that specific test. The number COVID-19-positive participants from our study sample (n=31) that would have been misidentified as COVID-19-negative are shown for each test based on the antibodies used against SARS-CoV-2 antigens (**Table S1**).

Loss of smell was more severe in COVID-19-positive participants

Several participants in both the COVID-19-positive and COVID-19-negative groups reported symptoms. Therefore, we analyzed differences in symptom presentation and severity between the 21 symptomatic participants that tested positive and the 18 symptomatic participants that tested negative using two-tailed Independent Samples T-Tests. All participants self-reported the first date they remembered experiencing symptoms, symptom severity, and the date on which the symptoms resolved. Days between

symptom onset and symptom resolution were not significantly different between the COVID-19-positive symptomatic participants and COVID-19-negative symptomatic participants (**Table S2**). Eighteen different symptoms were reported with varying severities between the symptomatic COVID-19-positive and COVID-19-negative participants (**Table S2**). Severity of loss of smell was the only symptom significantly increased in positive participants (mean(SE)=4.50±0.50) when compared to negative participants (mean(SE)=2.50±0.66) ($t(6)=-1.57$, $p<0.05$, **Table 1, Figure 1a**). These data suggest that severe loss of smell may be uniquely associated with SARS-CoV-2 infection. We ran Pearson's correlations with the total symptom severity scores for each symptom and the compiled total score and found that none of the 18 symptoms were significantly correlated with age or BMI (data not shown). There were no differences in symptom severity between males or females for any of the reported symptoms (data not shown).

Antibody titers against SARS-CoV-2 antigens in mild versus severe symptoms

Although no participants in the present study required hospitalization, both COVID-19-negative and COVID-19-positive participants reported experiencing symptoms that were severe (defined as a symptom that could not be ignored and was the worst the patient had ever felt) or moderate (defined as a symptom that could not be ignored and limited daily activities). To further investigate whether titer levels were elevated in participants with more severe symptomology overall, we compared antibody titers against SARS-CoV-2 antigens in COVID-19-positive participants ($n=21$) who reported having mild-moderate symptoms ($n=13$) and those who reported moderate-severe symptoms ($n=8$) on the questionnaire using two-tailed Independent Samples T-Tests. Antibody titers against SARS-CoV-2 antigens were compared in the 21 symptomatic COVID-19-positive participants (**Figure 1b**). Titers of most antibody/antigen combinations were elevated in COVID-19-positive participants with moderate-severe symptoms when compared to those with mild-moderate symptoms (data not shown). However, only titers of IgA antibodies against SARS-CoV-2 RBD were significantly higher in COVID-19-positive participants with moderate-severe symptoms (mean(SE)=1.00±0.23) when compared to participants with mild-moderate symptoms (mean(SE)=0.48±0.05), ($t(19)=-2.72$, $p<0.01$, **Figure 1b**).

IgM and IgG timelines

Next, we assessed the variation between reported symptom onset and serology test date. There were no significant correlations between titers and days between symptom onset and resolution or days between symptom onset and the initial test (Pearson's correlations, data not shown). However, within the COVID-19-positive population, subgroups of participants with high titers of antibodies stratified to distinct intervals after symptom onset (**Figure 2a-b**). COVID-19-positive participants displayed elevated levels of IgM and IgG (each immunoglobulin averaged for the four antigens tested) between 0-30 and 0-60 days, respectively, after symptom onset. IgM levels appeared to decline 30 days past symptom onset.

Based on the findings described above, we next compared all combinations of antibody titers against SARS-CoV-2 antigens in the 21 symptomatic COVID-19-positive participants at 0-30, 30-60, 60-90, and 90-120 days between symptom onset and the serology test date using one-way ANOVA tests. Average IgG antibody concentration was not significantly different between each interval, but was significantly higher between 0-30 days (mean=1.14) compared to 30-60 days (mean=1.03) (**Figure 2a**). The average levels of IgM antibodies against the S1 ($F(3,17)=10.89, p<0.001$; **Figure 2c**) and RBD ($F(3,17)=4.584, p<0.01$; **Figure 2d**) antigens were significantly different between the four intervals described above (**Figure 2c-d**). Specifically, there were significant differences between the 0-30 and 30-60 day intervals ($p<0.0001$) and the 0-30 and 60-90 day intervals ($p<0.001$) for the S1 antigen and the RBD. Furthermore, average IgM titers were significantly different between the 0-30 and 30-60 day intervals and between the 0-30 and 60-90 day intervals between symptom onset and the initial test ($F(3,17)=4.42, p<0.01$, **Figure 2b**). The 0-30 day interval had the highest average IgM concentration (mean(SE)= 1.08 ± 0.11), compared to the other three intervals.

Antibody titers in COVID-19-positive symptomatic versus asymptomatic participants

Differences in demographics and clinical characteristics between asymptomatic and symptomatic COVID-19-positive participants were examined next. There were no significant differences in sex, age, BMI, smoking history, or the presence of chronic illnesses between these two groups when analyzed by two-tailed Independent Samples T-Tests (**Table 3** and data not shown). Fisher's Exact Tests were used to compare the proportion of asymptomatic and symptomatic participants who tested positive for each antibody/antigen combination. We found that a greater number of symptomatic participants tested positive for IgG against the S1 antigen ($n=10, p<0.05$) and the NP antigen ($n=10, p<0.05$) when compared to asymptomatic COVID-19-positive participants ($n=0$ for both S1 and NP; **Table 3**). Similarly, more symptomatic participants tested positive for IgG against S2 ($n=12$) than asymptomatic participants ($n=1$), and this result trended towards significance ($p=0.08$, **Table 3**). The titers for the remaining antibody/antigen combinations were compared via two-tailed Independent Samples T-Tests and there were no significant differences between the two groups (**Table 3**).

Finally, we utilized average titers for IgA, IgG, and IgM against all antigens and examined differences in COVID-19-positive asymptomatic and symptomatic participants using two-tailed Independent Samples T-Tests. We found that the average IgG antibody titers against all antigens was significantly greater in COVID-19-positive symptomatic participants (mean(SE)= 1.04 ± 0.11) than COVID-19-positive asymptomatic participants (mean(SE)= 0.64 ± 0.10), ($t(26)=-2.00, p<0.05$). These data suggest that asymptomatic and symptomatic COVID-19-positive participants may experience different immune responses to SARS-CoV-2 infection. We utilized a Fisher's Exact Test to explore associations between IgA, IgG, or IgM antibody positivity and the presence or absence of symptoms. We found that a larger number of asymptomatic COVID-19-positive participants had at least one positive IgM antigen compared to the number with at least one positive IgG antigen ($p<0.05$, **Table 3**). Collectively, the above findings suggest

that COVID-19-positive symptomatic participants exhibited a greater IgG immune response and that asymptomatic participants had a greater IgM response. Intriguingly, none of the COVID-19-positive asymptomatic participants were positive for IgA against any viral antigen tested (**Table 3**).

Given the potential differences in disease progression in the asymptomatic and symptomatic participants positive for COVID-19, we wanted to understand whether there were differences in the antibody and antigen profile between asymptomatic participants, participants experiencing symptoms at the time of their serology test date (i.e., current symptomatic) and participants who had experienced symptoms before their test date (i.e., prior symptomatic). A Chi-square goodness of fit test showed that prior symptomatic COVID-19-positive participants were more likely to be positive for only IgG antibodies against any of the four SARS-CoV-2 antigens (n=7) than current symptomatic participants (n=1) or asymptomatic participants (n=2), ($\chi^2(1,2)=6.2$, $p<0.05$, **Table S3**). Although these sample sizes are quite small, these data further indicate that the majority of the participants in our cohort mounted a typical immune response to SARS-CoV-2 with IgM peaking early and IgG peaking later in the course of infection. Although the data were not significant, asymptomatic COVID-19-positive participants were more likely to be positive for only IgM antibodies against any of the four SARS-CoV-2 antigens (n=5; **Table S3**), than current or prior symptomatic participants (n=2; **Table S3**) suggesting a distinct immunoglobulin profile relative to symptom onset.

Effects of influenza vaccine on symptomatology

We also examined effects of a recent influenza vaccination (between January 1, 2018 and May 26, 2020) on SARS-CoV-2 symptoms and serology. A Chi-square test was used to analyze the relationship between receiving a recent influenza vaccination and testing positive or negative for COVID-19. Influenza vaccination questionnaire responses from 28 COVID-19-positive participants and 36 COVID-19-negative participants were analyzed. There was a significantly larger fraction of participants who tested positive for COVID-19 and had a recent influenza vaccination (n=18) than participants who tested negative (n=13) ($\chi^2(1,64)=5.01$, $p<0.05$, **Table 2**). These findings indicate that receiving the influenza vaccination had no effect on susceptibility to SARS-CoV-2 infection.

Next, we examined whether a recent influenza vaccination could attenuate symptomatology in COVID-19-positive participants. A Chi-square test was used to discern the relationship between asymptomatic and symptomatic participants who tested positive for COVID-19 and who received a recent influenza vaccination. We found that 11 positive symptomatic participants had a recent influenza vaccination compared to 7 positive asymptomatic participants. The 7 positive asymptomatic participants who received the influenza vaccination represent 100% of that group, whereas the 11 positive symptomatic participants who received the influenza vaccination constitute only 52% of that group ($\chi^2(1,28)=5.19$, $p<0.05$, **Table 3**). This result may indicate that the influenza vaccination provides protection against presentation or severity of COVID-19 symptoms. No significant effect was found for the BCG vaccine (data not shown). There were no significant differences between participants who received the influenza

vaccination and those who did not, regardless of test result or symptom presentation, in terms of titers, sex, age, BMI, or symptom severity (**Table S4**).

Discussion

In the current study, we utilized a novel serology test for COVID-19 in an otherwise healthy cohort of adults and children in Colorado. Recent results using the same test kit have shown that IgM antibodies against SARS-CoV-2 are generally detectable in blood several days after initial infection, although levels over time are not well characterized²⁶. Our results show that IgM levels and IgG levels were both elevated early (0-30 days following symptom onset). IgM levels declined 30 days following symptom onset while IgG levels remained elevated for up to 60 days following symptom onset. Increased early IgM levels may indicate acute infection and a later elevation in IgG levels correspond to a prolonged immune response and the activation of adaptive humoral immunity⁸. Suhandynata and collaborators (2020)²⁹ demonstrated the evolution of seroconversion for both IgG and IgM in a cohort of acutely ill patients, supporting our findings here. Although IgG levels declined after 60 days post- symptom onset, a recent study indicates that relatively low levels of IgG may still provide significant immunity against SARS-CoV-2³⁰.

Contrary to these results, several studies have shown that IgM levels peaked around day 20 and fell below baseline by about 6 months, while IgG levels remained elevated beyond 2 years post- infection in SARS-CoV-1 infected patients^{22,24}. Despite the similarities between SARS-CoV-1 and SARS-CoV-2, it is possible that IgG levels do not remain elevated long-term for COVID-19 patients. Recently, several other studies have reported that IgG levels against SARS-CoV-2 were still elevated in COVID-19-positive patients after 49-50 days³¹. Similar results were described by Zhang et al. (2020), but there is little research on the long-term titers of IgG in a larger cohort²⁵. Recurrence of COVID-19 appears to be uncommon, suggesting that antibody presence could confer at least short- term immunity. This is in agreement with the findings of Chandrashekar et al. (2020) which showed that primates infected with SARS-CoV-2 were protected from reinfection following viral clearance³². Interestingly, IgG antibody development against SARS-CoV-2 has also been associated with a reduced viral load in the respiratory tract³³. These findings may indicate antibody development offers some level of protection from reinfection. Furthermore, it is generally accepted that patients with severe illness are more likely to have heightened immune responses and robust long-term immunity than those with milder illness³⁴. Thus, it should be noted that the cohort studied here represents a relatively young, healthy population which did not require hospitalization. It is possible that the decline in IgG levels may be due to a less robust immune response to a relatively mild infection^{12,19,35}. Serology profiles may look different in an elderly population, or those that have been primed by previous or chronic infectious diseases or conditions.

The current study contained a comprehensive symptom inventory, including respiratory symptoms, neurological symptoms, and GI-related symptoms (see **Table S2**). When comparing symptomology between COVID-19-positive and COVID-19-negative participants, we hoped to identify symptoms that were uniquely associated with COVID-19 infection and that correlated with immune profiles. Levels of

most antibody/antigen combinations were elevated in COVID-19-positive participants with moderate-severe symptoms when compared to participants with mild-moderate symptoms. The only symptom unique to positive participants was loss of smell. In a study by Dell’Era et al. (2020)³⁶, investigators examined the loss of taste and smell in a cohort of Italian patients with confirmed COVID-19. They found that 70% of this population experienced loss of taste or smell. In line with our findings, Menni et al. (2020) showed that out of 10 commonly reported symptoms of COVID-19, loss of smell and taste were the best predictors of a positive test result³⁷. It has been reported that loss of taste and smell may be the earliest signs of infection and thus, may represent a marker of viral shedding and a reliable predictor of infection.

We found that the average IgG antibody titers against all antigens were significantly greater in COVID-19-positive symptomatic participants than COVID-19-positive asymptomatic participants. Therefore, asymptomatic and symptomatic COVID-19-positive participants may exhibit different immune responses to SARS-CoV-2. Asymptomatic participants were more likely to test positive for IgM only. Interestingly, none of the asymptomatic participants were positive for IgA and participants whose symptomology was classified as moderate-severe had significantly higher titer levels of RBD IgA. IgA is the principal antibody in secretions including the mucus epithelium and the intestinal and respiratory tract and acts primarily as a neutralizing antibody to eliminate pathogens before infection begins³⁸. Neutralizing antibodies such as IgA typically block viral binding to surface receptors on cells and disable receptor-virus interaction. In addition, it has been reported that circulating IgA may be involved in the formation of immune complexes that amplify pro-inflammatory cytokine signaling³⁸. Several recent studies have found high levels of serum RBD IgA in COVID-19 patients that are significantly correlated with symptom severity^{39,40}. IgA may play a critical role in mucosal and systemic responses to SARS-CoV-2 infection and titer levels may provide an early indication of symptom severity and disease progression. Further research should be focused on characterizing the IgA response in COVID-19 patients with moderate-severe symptomology.

Trained immunity, or non-specific enhancement of the innate immune system that “primes” innate immune cells to respond quickly to novel pathogens is a well-described phenomenon in the virology field^{25,27,41}. One reason older individuals are especially vulnerable to SARS-CoV-2 infection could be that the aging process leads to increased reactivity to immune challenges, such that priming of the immune system is less effective⁴². This could also be why children are less susceptible to severe symptoms after SARS-CoV-2 infection. It has been suggested by others that childhood vaccinations might result in trained immunity of innate immune cells, so-called immune fitness or cross-protection^{43,44}. Therefore, innate immune cells may exhibit a heightened “ready state” in individuals who receive recent vaccinations, such as the influenza vaccine. Our findings do not suggest that the influenza vaccine protects individuals against infection, but instead might lead to milder symptoms. Whether the influenza vaccine leads to a more general ramping up of the immune defense in specific immune cells is not yet known and should be the target of future studies. The COVID-19-positive asymptomatic participants in this study all had a recent influenza vaccination and were more likely to exhibit an IgM response. This finding supports the idea that the influenza vaccination may enhance innate immune activation and alleviate severe symptomology in these participants. Since trained immunity is thought to

be a transient effect²⁷, it is logical that we found a significant effect of a recent, seasonal influenza vaccination and not the BCG vaccine, which is typically administered directly after birth⁴⁵.

There are several limitations to the present study. First, the sample sizes are relatively small and the population was largely young and healthy. Future studies should focus on a more diverse population of participants with moderate-severe COVID-19 symptomology. Participants in this study were not uniformly subjected to PCR testing to confirm SARS-CoV-2 infection. In addition, serological testing was only performed at one time-point and with varied duration from symptom onset. Nonetheless, the data presented are still valuable in identifying unique COVID-19 symptomology and characterizing the immune response to SARS-CoV-2. Our findings confirm those of previous studies and provide new insight regarding the immune response in symptomatic versus asymptomatic patients. Furthermore, this study is the first to present the novel idea of immune system priming to SARS-CoV-2 associated with the influenza vaccination.

In conclusion, the data presented herein indicate that IgM antibody levels were elevated earlier in the course of symptoms in participants with a positive serological test result, and that IgG levels tended to be elevated longer than IgM. IgM appeared to decline below positive-detection levels 30 days post-symptom onset. The average IgG antibody titers against all antigens were significantly greater in COVID-19-positive symptomatic participants than in asymptomatic participants. COVID-19- positive symptomatic participants exhibited a greater IgG immune response while asymptomatic participants had a greater IgM response. These findings could aid in distinguishing individual immune responses in a wider population for tracking purposes. Our data further suggest elevated IgA levels may track with increased symptom severity and that the influenza vaccine does not provide protection against SARS-CoV-2 infection, but may attenuate symptom severity.

Online Methods

Participants

One hundred eight non-hospitalized participants (n=108) were recruited from a community clinic designated for the care of patients without severe or life-threatening symptoms of COVID-19. All study participants underwent a detailed history by a board certified physician and filled out an online questionnaire (detailed below). Participants were excluded from the study if they were not permanent residents of Colorado or if they reported symptoms before December 25, 2019. Symptomatic and asymptomatic participants were deemed probable COVID-19 cases based on clinical presentation and/or prior known or suspected exposure to an individual with a PCR-confirmed case of COVID-19. Blood serum samples were collected from each patient as detailed below. A total of thirty-one participants (n=31) had positive antibody tests, defined by a titer concentration ≥ 1 (as defined below) for at least one antibody/antigen combination, and were deemed confirmed COVID-19 cases. Each participant in this cohort was further categorized into two groups (either “asymptomatic” or “symptomatic”) based on their self-reported symptoms or lack of symptoms. The positive symptomatic group was comprised of both

current symptomatic (participants who were symptomatic on the test date) and prior symptomatic participants (whose symptoms had resolved before the test date). This information was extracted from questionnaire responses as described below. The remaining seventy- six participants (n=76) with negative antibody tests were also categorized into two groups (either “asymptomatic” or “symptomatic”) based on their self-reported symptoms or lack of symptoms. Demographic information for the study population is shown in **Table 1**.

Questionnaire

Demographic information, medical histories, questions related to SARS-CoV-2 exposure and prior testing, vaccinations, travel, physical activity levels, drug use, and date of symptom onset, resolution and severity were gathered using a 42-item electronic form that took an average of 10-15 minutes to complete. A total of 65 participants provided questionnaire responses. Sixty-three (n=63) filled out the form an average of 18 days (median=16 days; range=3-48 days) following their initial serology antibody test. Two (n=2) participants responded to the questionnaire 2 and 9 days prior to their initial serology antibody test (average=6 days). Presence of specific symptoms and symptom severity were reported on a 5-point Likert scale with 0 indicating the absence of the specific symptom, 1 being a mild symptom, and 5 being a severe symptom. Reported symptoms included fever, dry cough, sore throat, fatigue, sputum production, nasal congestion, runny nose, headache, loss of smell, loss of taste, vomiting, diarrhea, dizziness, chills, body aches or myalgia, shortness of breath, swollen lymph nodes, and chest pain. A total symptom severity score was generated by adding the severity score for each of the 18 individual symptoms. The total possible symptom severity score was 90. Participants were asked to report the presence of illness between December 25, 2019 and May 26, 2020 and reported the first date they remembered experiencing symptoms and the date on which the symptoms resolved. We defined the course of illness as the period from the onset of symptoms to the date of symptom resolution.

Blood collection

Blood samples were obtained from all 108 participants. Each blood sample was taken between 1-117 days of symptom onset if the participant was experiencing symptoms. A certified phlebotomist performed each venipuncture at Resilience Code (Englewood, Colorado). From each participant and for each draw, 7.5 mL of blood was drawn from the antecubital vein and collected in one VACUETTE® serum separator tube (SST) (Greiner Bio-One, Monroe NC) containing a clot activator and gel which was provided in the chemiluminescence immunoassay (CLIA) kit supplied by Vibrant (Vibrant COVID-19 Ab Assay; Vibrant America Clinical Labs). Each SST tube was allowed to clot at room temperature (approximately 20°C) for 30 minutes. Serum separator tubes were then centrifuged at 3000 RPMs at room temperature for 20 minutes. Samples were stored at Resilience Code at room temperature for no longer than 8 hours prior to shipping the same day or were refrigerated and stored at 4°C prior to shipping to Vibrant America Clinical Labs (San Carlos, CA) the day following blood collection. Dates and times of

blood draws, duration between blood draw and shipping, and duration between blood draw and reported results were recorded.

Serological testing

Serological testing was performed by Vibrant America using a SARS-CoV-2 IgM, IgG and IgA CLIA (Vibrant COVID-19 Ab Assay; Vibrant America Clinical Labs). This assay was approved by the FDA for Emergency Use Authorization (<https://www.fda.gov/medical-devices/emergency-situations-medical-devices/eua-authorized-serology-test-performance>). The assay protocol was as previously described²⁶. Microbial contaminated or specimens containing visible particulate were excluded. Grossly hemolyzed or lipemic serum or specimens were avoided. The samples were stored at 2-8°C for up to 7 days before assay. Briefly, purified recombinant SARS-CoV-2 antigens, including S1 subunit of Spike Protein (S1), Receptor Binding Domain (RBD), S2 subunit of Spike Protein (S2) and the Nucleoprotein (NP), were bound to functionalized silicon chips and assembled onto a 96-pillar plate. A layout of 8 chips on each pillar (4 chips with SARS-CoV-2 antigens and 4 reference chips used in software analysis) was created using an automated semiconductor assembly technique. The assay was performed using three 96 pillar plates for each assay (one for IgG antibody detection, one for IgA antibody detection and one for IgM antibody detection) and an automated liquid handling workstation (Hamilton Microlab STAR). After blocking, the positive control, negative control, cut-off control, and diluted patient sera (1:50) were added to the wells and allowed to incubate for 15 minutes at room temperature. The plates were washed 3 times with 1X Tris-Buffered Saline containing 0.1% Tween® 20 Detergent (TBST) buffer (Amresco INC, Solon OH) for 5 minutes each time to remove any unbound sample. A 1:2000 dilution of Goat Anti-Human IgG HRP, Goat Anti-Human IgM HRP and Goat Anti Human IgA HRP secondary antibody was then added individually for 15 minutes at room temperature. After washing with TBST and DI H₂O, remaining enzyme activity was measured by adding a chemiluminescent substrate (Clarity Max from Bio-Rad, Hercules CA). The intensity of the signal from each chip was measured using a high resolution chemiluminescence imager (Q-View™ Imager Pro, Quansys Biosciences, Logan UT). Each plate was scanned for 5 minutes. A reporter software was used to obtain the raw chemiluminescent signals.

The raw chemiluminescent signals were subjected to quantile normalization, spatial correction, and background correction. The mean \pm SD of the signal intensity for each antigen was calculated from healthy controls used as the signal threshold. The raw sample results were quantitated into arbitrary chemiluminescent units by comparison with cut-off values. A sample cohort of 368 samples which consist of healthy controls collected prior to the SARS-CoV-2 outbreak was used to determine the cut-off. The upper 97th percentile was set to 1.00 for each antigen tested. Participant samples were considered to be negative for COVID-19 if the sample intensity was equal to or less than 1.00 and positive for COVID-19 if it was greater than 1.00.

Data cleaning and statistical analysis

Analysis was conducted using data from a total of one hundred and eight (n=108) participants. For comparison of ethnicity, BMI, smoking history (tobacco or marijuana), hypertension, cardiovascular disease, diabetes, liver disease, autoimmune disease, tick borne illness, hemochromatosis, blood related disease (i.e., anemia), vaccination history, and symptom severity, data was used from a total of sixty-five (n=65) out of the one hundred and eight (n=108) participants who responded to the questionnaire. One participant was missing titer information and was not determined to be COVID-19 positive or negative but was included in analyses involving the variables described above. No outliers were removed from the data set prior to analysis.

Exploratory correlational analyses were performed using the total study population and within subgroups of participants who tested either positive or negative for SARS-CoV-2. Pearson's correlations were used to investigate possible relationships between demographic variables (age and BMI) and the compiled severity scores for the 18 COVID-19 symptoms that were included on the questionnaire. The data were adjusted for multiple comparisons using the Holm procedure to avoid inflation of the Type 1 error rate.

To further investigate the relationship between symptom severity and titer concentrations, a categorical variable was generated using a median split of the total symptom severity score for positive symptomatic participants. The median symptom severity score was 27. Participants who reported a total symptom severity score equal to or below 27 were categorized as having mild- moderate symptoms while those who reported a score above 27 were categorized as having moderate-severe symptoms. Two-tailed Independent Samples T-tests were used to determine differences in levels of all 12 of the antibody/antigen combinations and the two symptom severity groups.

To test for intergroup differences in the number of subjects with positive antibody/antigen combinations, certain symptoms, a recent influenza vaccination, and demographic variables (age, BMI, gender, medical conditions, smoking history, etc.) two-tailed Independent Samples T-Tests, Chi Square Analyses or Fisher's Exact Tests were utilized. Two-tailed Independent Samples T-tests were used for continuous data. Chi Square analyses were used with categorical data that had more than 5 cases in all cells of the generated contingency tables, and Fisher's Exact Test was used with categorical data that had less than 5 cases in any one of the cells. A Chi Square Goodness of Fit Test was used to test for differences in the number of subjects that tested positive for IgG or IgM antibodies with one observed variable.

A categorical variable was generated using the calculated days between symptom onset and the initial test to look at possible differences in titers at certain time intervals between symptom onset and the initial test. Separate one-way ANOVAs were used to test whether mean titers differed between the four intervals (0-30 days, 30-60 days, 60-90 days, 90-120 days) from symptom onset to the initial test. Tukey post hoc analyses were conducted to evaluate differences between adjusted means.

Thirteen SARS-CoV-2 antibody detection assays approved for Emergency Use Authorization by the FDA were compared to the Vibrant COVID-19 Ab Assay used in the present study (U.S. FDA, 2020). The antibody and SARS-CoV-2 antigens detected by each test were recorded. We calculated the number of COVID-19 participants in our sample (n=31) that were not positive for the antibody antigen combination

detected by each assay. This number was expressed as a percentage of positive missed out of our total sample population of COVID-19 positive participants. All statistical analyses and data visualization were performed using RStudio for Mac (RStudio Team [2019]. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL). The alpha level for null hypothesis rejection was set at 0.05. Data are presented as mean \pm standard error, unless otherwise noted.

Declarations

Ethical approval and informed consent process

Each patient granted Resilience Code specific, written authorization to disclose their medical records for research purposes. This study was determined to be exempt from IRB approval by the IRB at the University of Denver. All protected health information (PHI) was de-identified prior to analysis. A random global unified identifier (GUID) code was assigned to each participant and was used on all samples and forms associated with the study to maintain anonymity.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. Raw serological testing data were generated at Vibrant Clinical Labs and is not publicly available.

Code Availability

Computer code used to analyze the data can be requested from the corresponding author on reasonable request. The software and computer code used to analyze the raw data from the CLIA assay is the property of Vibrant Clinical Labs and is not publicly available.

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Author Contributions

D.A.L., A.C.G., C.P. supervised and conceptualized this project. C.P. recruited participants for this study and executed clinical protocols. H.K.K and V.J. developed the FDA Emergency Use Authorization assay, processed clinical samples, and performed SARS-CoV-2 serological testing. Research data was maintained and statistically analyzed by A.N.G, L.A.K. The original draft was written by A.C.G., A.N.G, L.A.K. The text was edited by A.C.G, D.A.L., A.L., A.N.G., L.A.K. with input from C.P., H.K.K., and V.J.

Competing Interests statement

H.K.K. and V.J. are affiliated with Vibrant America Clinical Labs, a commercial lab that performs commercial antibody testing for the novel coronavirus. All other authors declare no competing interests.

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Tables

Table 1. Demographic and clinical characteristics of the total population, COVID-19-positive and COVID-19-negative participants. Population percentages for ethnicity, smoking history (tobacco or marijuana), hypertension, cardiovascular disease, diabetes, liver disease, autoimmune disease, tick borne illness, hemochromatosis, blood related disease (i.e., anemia), and loss of smell were calculated based on the number of total (n=65), COVID-19-positive (n=28), and COVID-19- negative (n=36) participants who responded to the questionnaire. Data are presented as the mean (SE), range, or number (%). Statistical differences are shown for COVID-19-positive versus COVID- 19-negative participants. *p*-values were determined via two-tailed Independent Samples T-Tests.

	Total Study Population	COVID-19-Positive	COVID-19-Negative	p-Value
Sample Size (n)	108	31	76	-
Age, years	42.33 (1.52) Range = 12 - 78	40.74 (2.75) Range = 12 - 68	42.73 (1.84) Range = 12 - 78	ns
Male sex	53 (49.07%)	12 (38.71%)	40 (52.63%)	ns
Ethnicity	White (62) (95.38%) Asian (2) (3.08%) Hispanic, Latino or Spanish (1) (1.54%)	White (25) (89.29%) Asian (2) (7.14%) Hispanic, Latino or Spanish (1) (3.57%)	White (36) (100.00%)	ns
BMI	25.19 (0.55) Range = 18.56 - 43.26	25.21 (0.84) Range = 20.22 - 43.26	25.17 (0.75) Range = 18.56 - 41.05	ns
Smoking history (tobacco or marijuana)	10 (15.38%)	4 (14.29%)	6 (16.67%)	ns
Hypertension	0 (0.00%)	0 (0.00%)	0 (0.00%)	ns
Cardiovascular Disease	4 (6.15%)	0 (0.00%)	4 (11.11%)	ns
Diabetes	1 (1.53%)	1 (3.57%)	0 (0.00%)	ns
Liver disease	0 (0.00%)	0 (0.00%)	0 (0.00%)	ns
Autoimmune Disease	5 (7.69%)	2 (7.14%)	3 (8.33%)	ns
Tick Borne Illness	7 (10.77%)	2 (7.14%)	5 (13.89%)	ns
Hemochromatosis	1 (1.54%)	1 (3.57%)	0 (0.00%)	ns
Blood related disease (I.e., anemia)	3 (4.62%)	2 (7.14%)	1 (2.78%)	ns
Loss of smell	3.22 (0.55) Range = 1-5 9 (13.85%)	4.50 (0.50) Range= 3-5 4 (14.29%)	2.50 (0.66) Range= 1-4 4 (11.11%)	p < 0.05

Table 2. Comparison of IgM, IgA, and IgG titers against SARS-CoV-2 spike 1 glycoprotein (S1 SP), spike 2 glycoprotein (S2 SP), receptor binding domain (RBD), and nucleoprotein (NP) in COVID-19-positive and COVID-19-negative participants. Duration between symptom presentation and initial serological testing are displayed for symptomatic COVID-19-positive (n=21) and COVID-19-negative participants (n=18) who filled out the questionnaire. The number of participants out of the total population with titer data (n=107) that are negative for each individual antibody and titer are displayed in the COVID-19-negative column. Data are presented as the mean (SE), range, or number (%). p-values were determined via two-tailed Independent Samples T-Tests. + indicates participants positive for the antibody/antigen combination.

	COVID-19-Positive	COVID-19-Negative	<i>p</i> -Value
Sample Size (n)	31	76	-
Days between symptom onset and initial serological test	44.43 (6.68) Range = 6 - 117	56.41 (7.89) Range = 1 - 114	ns
Influenza Vaccination (Yes)	18 (64.9%)	13 (36.11%)	<i>p</i> < 0.05
S1 SP IgM +	12 (38.71%)	-	-
S1 SP IgM titer, AU/ml	1.50 (0.08) Range = 1.06 - 1.90	0.58 (0.02) Range = 0.24 - 0.87	<i>p</i> < 0.01
S1 SP IgG +	12 (38.71%)	-	-
S1 SP IgG titer, AU/ml	1.61 (0.11) Range = 1.09 - 2.24	0.53 (0.02) Range = 0.22 - 0.87	<i>p</i> < 0.01
S1 SP IgA +	3 (9.68%)	-	-
S1 SP IgA titer, AU/ml	1.60 (0.28) Range = 1.05 - 1.94	0.54 (0.02) Range = 0.22 - 0.87	<i>p</i> < 0.01
RBD IgM +	8 (25.81%)	-	-
RBD IgM titer, AU/ml	1.48 (0.13) Range = 1.11 - 2.08	0.57 (0.02) Range = 0.24 - 0.87	<i>p</i> < 0.01
RBD IgG +	13 (41.94%)	-	-
RBD IgG titer, AU/ml	1.67 (0.10) Range = 1.17 - 2.26	0.55 (0.02) Range = 0.22 - 0.87	<i>p</i> < 0.01
RBD IgA +	5 (16.13%)	-	-
RBD IgA titer, AU/ml	1.53 (0.16) Range = 1.15 - 2.11	0.53 (0.02) Range = 0.22 - 0.86	<i>p</i> < 0.01
S2 SP IgM +	11 (35.48%)	-	-
S2 SP IgM titer, AU/ml	1.41 (0.08) Range = 1.08 - 1.99	0.50 (0.02) Range = 0.22 - 0.86	<i>p</i> < 0.01
S2 SP IgG +	15 (48.39%)	-	-
S2 SP IgG titer, AU/ml	1.62 (0.11) Range = 1.05 - 2.21	0.56 (0.02) Range = 0.23 - 0.87	<i>p</i> < 0.01
S2 SP IgA +	6 (19.35%)	-	-
S2 SP IgA titer, AU/ml	1.55 (0.09) Range = 1.28-1.83	0.55 (0.02) Range = 0.27 - 0.85	<i>p</i> < 0.01
NP IgM +	10 (32.26%)	-	-
NP IgM titer, AU/ml	1.49 (0.10) Range = 1.04 - 2.07	0.56 (0.02) Range = 0.22 - 0.86	<i>p</i> < 0.01
NP IgG +	12 (38.71%)	-	-
NP IgG titer, AU/ml	1.681667 (0.11) Range = 1.08 - 2.27	0.54 (0.02) Range = 0.23 - 0.86	<i>p</i> < 0.01
NP IgA +	3 (9.66%)	-	-
NP IgA titer, AU/ml	2.40 (0.82) Range = 1.24 - 3.99	0.57 (0.02) Range = 0.24 - 0.87	<i>p</i> < 0.01

Table 3. Comparison of IgM, IgA, and IgG titers against SARS-CoV-2 spike 1 glycoprotein (S1 SP), spike 2 glycoprotein (S2 SP), receptor binding domain (RBD), and nucleoprotein (NP) in symptomatic and asymptomatic COVID-19-positive participants. Age, sex, recipient of the influenza vaccine, and BMI are also shown for comparison in symptomatic (n=21) and asymptomatic (n=7) COVID-19-positive participants. Data are presented as the mean (SE), range, or number (%). *p* values were determined via two-tailed Independent Samples T-Tests for continuous data, Chi-Squared Tests for categorical data with >5 cases per cell and Fisher's Exact Tests for categorical data with <5 cases per cell. Abbreviations: + indicates participants positive the antibody/antigen combination.

	COVID-19-Positive: Symptomatic	COVID-19-Positive: Asymptomatic	<i>p</i> -Value
Sample Size (n)	21	7	
Age	38.48 (3.25) Range = 12-62	37.71 (4.30) Range = 26-53	ns
Male sex	8 (38.10%)	1 (14.29%)	ns
Influenza Vaccination (Yes)	11 (52.38%)	7 (100.00%)	<i>p</i> < 0.05
BMI	25.42 (1.11) Range = 20.22 - 43.26	24.57 (0.68) Range = 21.79 - 27.12	ns
S1 SP IgM +	7 (33.33%)	3 (42.86%)	ns
S1 SP IgM titer, AU/ml	1.56 (0.11) Range = 1.29 - 1.90	1.35 (0.16) Range = 1.06 - 1.59	ns
S1 SP IgG +	10 (47.62%)	0 (0.00%)	<i>p</i> < 0.05
S1 SP IgG titer, AU/ml	1.58 (0.13) Range = 1.09 - 2.24		
S1 SP IgA +	2 (9.52%)	0 (0.00%)	ns
S1 SP IgA titer, AU/ml	1.43 (0.39) Range = 1.05 - 1.82		
RBD IgM +	5 (23.81%)	2 (28.57%)	ns
RBD IgM titer, AU/ml	1.59 (0.19) Range = 1.11 - 2.08	1.31 (0.02) Range = 1.29 - 1.33	ns
RBD IgG +	10 (47.62%)	1 (14.29%)	ns
RBD IgG titer, AU/ml	1.57 (0.10) Range = 1.17 - 2.14	1.92 (NA) Range = 1.92 - 1.92	ns
RBD IgA +	4 (19.05%)	0 (0.00%)	ns
RBD IgA titer, AU/ml	1.52 (0.21) Range = 1.15 - 2.11		
S2 SP IgM +	7 (33.33%)	3 (42.86%)	ns
S2 SP IgM titer, AU/ml	1.43 (0.12) Range = 1.08 - 1.99	1.39 (0.05) Range = 1.29 - 1.47	ns
S2 SP IgG +	12 (57.14%)	1 (14.29%)	<i>p</i> = 0.08
S2 SP IgG titer, AU/ml	1.57 (0.12) Range = 1.05 - 2.21	2.04 (NA) Range = 2.04 - 2.04	ns
S2 SP IgA +	5 (23.81%)	0 (0.00%)	ns
S2 SP IgA titer, AU/ml	1.53 (0.11) Range = 1.28 - 1.83		
NP IgM +	5 (23.81%)	4 (57.14%)	ns
NP IgM titer, AU/ml	1.51 (0.13) Range = 1.04 - 1.79	1.45 (0.21) Range = 1.18 - 2.07	ns
NP IgG +	10 (47.62%)	0 (0.00%)	<i>p</i> < 0.05
NP IgG titer, AU/ml	1.68 (0.14) Range = 1.08 - 2.27		
NP IgA +	3 (14.29%)	0 (0.00%)	ns
NP IgA titer, AU/ml	2.40 (0.82) Range = 1.24 - 3.99		

Figures

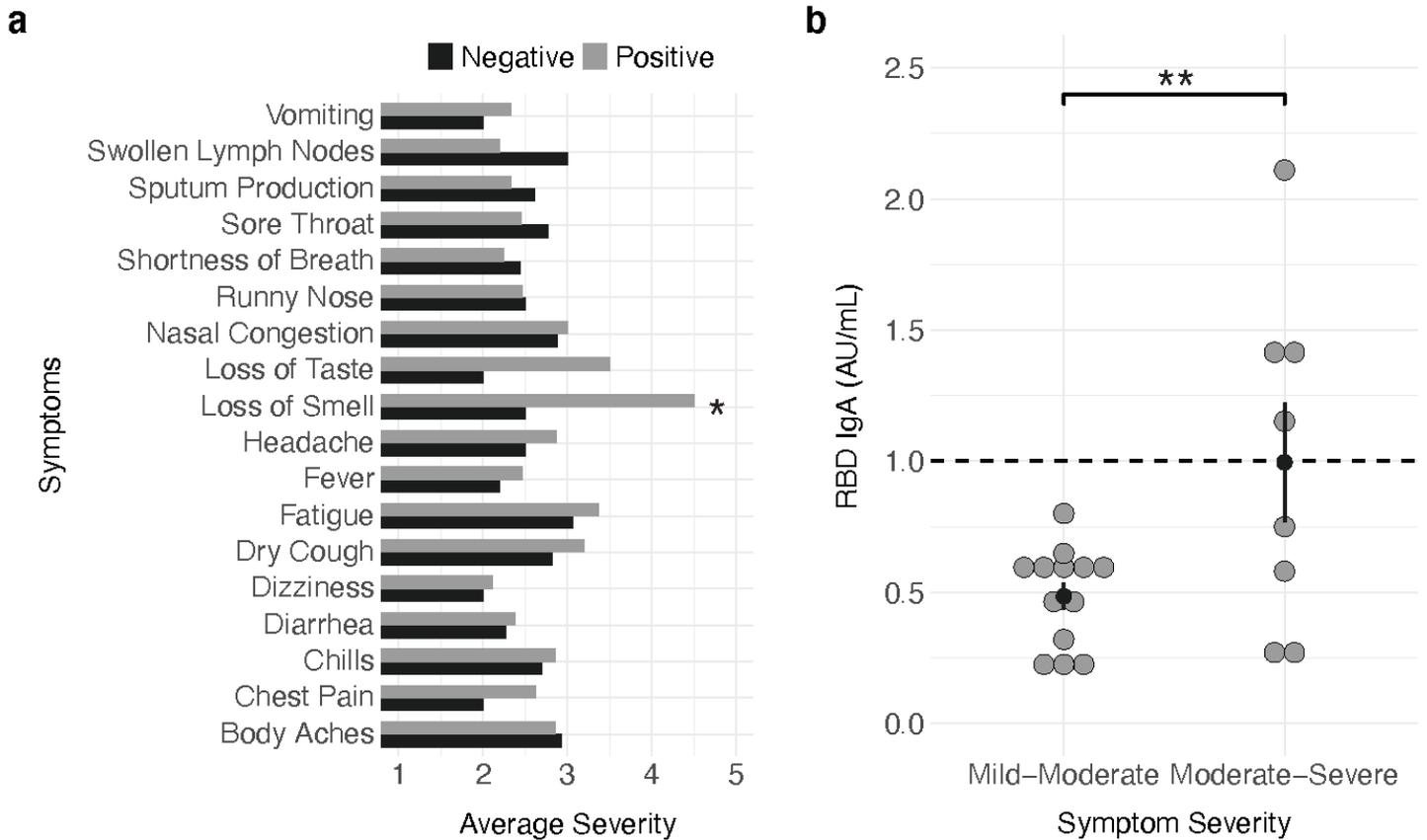


Figure 1

Symptom severity and relationship to antibody titers in COVID-19-positive and COVID-19-negative participants. Participants who reported being ill between January 1, 2020 and May 26, 2020 were asked to report the presence of specific symptoms experienced during their illness and the severity of each symptom on a 5-point Likert scale (1=mild, 5=severe). An average symptom severity score was calculated for each participant. Those with an average symptom severity of less than 2.5 were considered to have a mild-moderate case and those who reported an average symptom severity greater than 2.5 were considered to have a moderate-severe case. (a) Symptom presentation and severity for symptomatic COVID-19-positive and COVID-19-negative participants. Self-reported presentation and severity of 18 symptoms in symptomatic COVID-19-positive (n=21; grey) and COVID-19-negative (n=18; black) participants. Loss of smell severity was significantly increased in COVID-19-positive participants (mean(SE)=4.50±0.50) compared to COVID-19-negative participants (mean(SE)=2.50±0.66), $t(6)=-1.57$, $p<0.05$, * indicates $p<0.05$. Data are presented as the mean severity for each symptom. (b) Levels of IgA titers against SARS-CoV-2 receptor binding domain (RBD) plotted against symptom severity. COVID-19-positive participants who tested positive for IgA RBD with moderate-severe COVID-19 symptomology (n=8) (mean(SE)=1.00±0.23) had significantly higher levels than those with mild-moderate symptomology (n=13) (mean(SE)=0.48±0.05), $t(19)=-2.72$, ** indicates $p<0.01$. Data are presented as the mean and error bars represent SE. The cut-off value for the serological test (≥ 1) is shown as a black dashed line for reference. All data were analyzed using two-tailed Independent Samples T-tests.

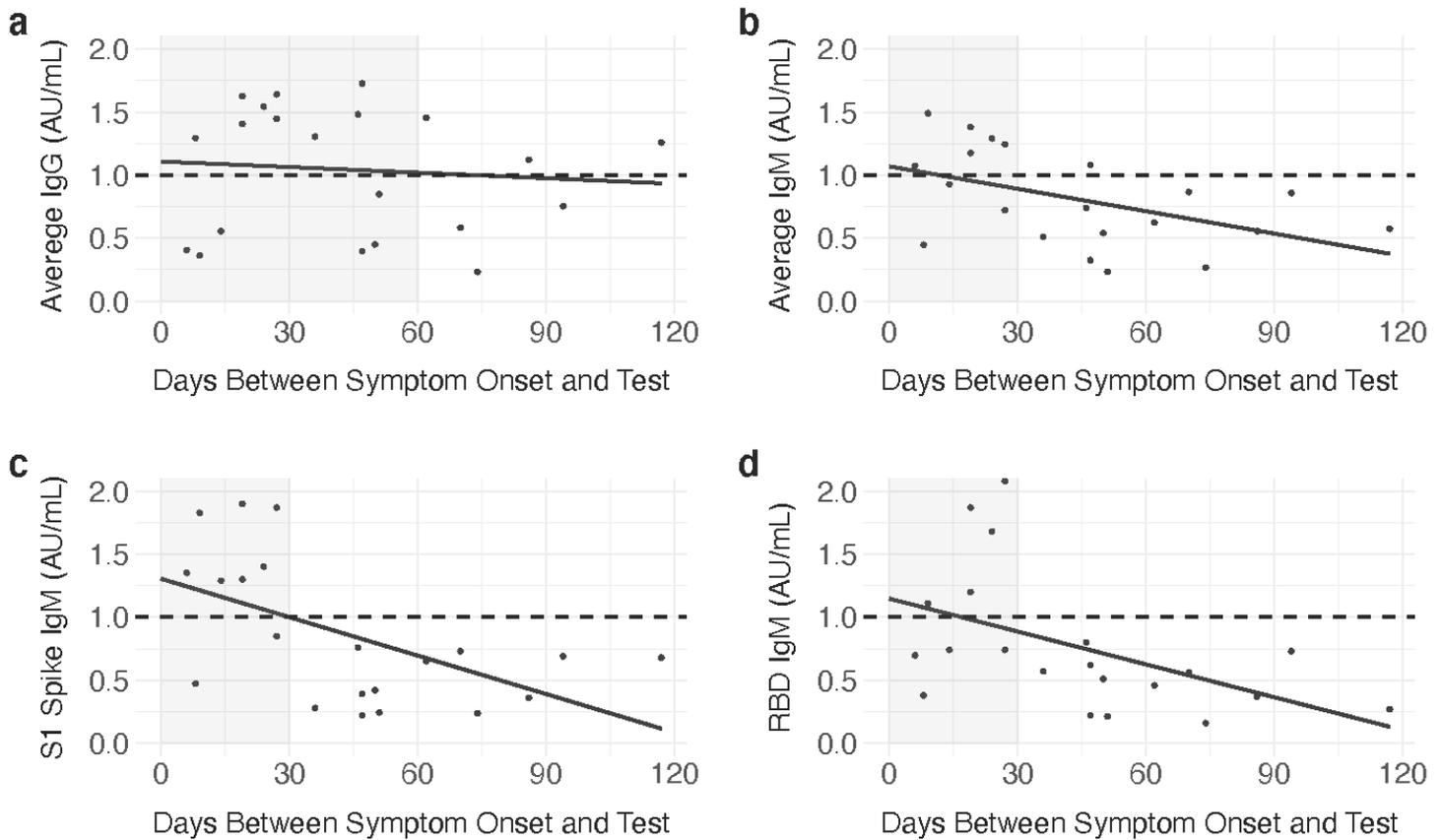


Figure 2

IgG and IgM antibody titers plotted against days between symptom onset and the initial serological test for COVID-19-positive participants. Includes symptomatic COVID-19-positive ($n=21$; black symbols and line) participants. a) Average IgG antibody titers were not significantly different between each interval, but were significantly elevated between 0-30 days (mean=1.14) compared to 30-60 days (mean=1.03). b) Average IgM antibody titers were significantly different between the 0-30 and 30-60 day intervals and between the 0-30 and 60-90 day intervals between symptom onset and the initial test ($F(3,17)=4.42$, $p<0.01$, Figure 2b). c) The average levels of IgM antibodies against the spike 1 glycoprotein (S1 Spike) were significantly different between 0-30, 30-60, 60-90, and 90-120 days ($F(3,17)=10.89$, $p<0.001$). Specifically, there were significant differences between the 0-30 and 30-60 day intervals ($p<0.0001$). d) The average levels of IgM antibodies against the receptor binding domain (RBD) were significantly different between 0-30, 30-60, 60-90, and 90-120 days ($F(3,17)=4.584$, $p<0.01$). Specifically, there were significant differences between the 0-30 and 60-90 day intervals ($p<0.001$). a,b) Average titers of both IgG and IgM were highest between 0-30 days. For IgG, titers were highly elevated, above the cut-off, in both the 0-30 and 30-60 day intervals (shown in grey shading). In comparison, IgM levels for all four antigens were highly elevated, above the cut-off, only between the 0-30 day intervals. The cut-off value for the serological test (>1) is shown as a black dashed line for reference. All data were analyzed using one-way ANOVA. Tukey post hoc tests were used for multiple comparisons.

Supplementary Files

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