Evaluation of the humoral immune response as a potential indicator of disease severity for patients hospitalized with COVID-19

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“Evaluation of the humoral immune response as a potential indicator of disease severity for patients hospitalized with COVID-19.”

Spencer Sterling, MPH – Epidemiology

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Abstract

Members of the family Coronaviridae, including the severe acute respiratory syndrome coronaviruses (SARS-CoV-1 and SARS-CoV-2), Middle East respiratory syndrome coronavirus (MERS-CoV) and seasonal human coronaviruses (HCoV-HKU1 and HCoV-OC43), are highly communicable respiratory viruses; SARS-CoV-1, SARS-CoV-2, and MERS can cause severe disease, while seasonal human coronaviruses present with mild to moderate illness. Coronavirus envelope spike (S) glycoproteins are involved in receptor binding and cell fusion, and are the primary target for a neutralizing humoral response. The S protein is comprised of the S1 and S2 subunits; the S1 subunit contains the receptor-binding domain (RBD), while the S2 subunit mediates fusion and cell entry. A conformational native S protein exists as a membrane-anchored S protein trimer. Stabilized pre-fusion S protein ectodomain (S-2P) trimers have been developed as vaccine candidates for SARS-CoV-1, SARS-CoV-2, and MERS-CoV (Corbett). Here, we investigated the humoral immune response in COVID-19 patients using a serological assay based on coronavirus S-2P trimers. Response kinetics were characterized, as well as associations between the magnitude of the response and prior HCoV exposure with disease severity and outcomes. Significant trends were observed that may be important in efficacious vaccine design and serological surveillance.
Chapter 1 – Introduction

Research question

Here, we wished to determine the prevalence and magnitude of reactive antibodies to SARS-CoV-2 and related coronaviruses in a cohort of patients enrolled at military treatment facilities with suspected or confirmed COVID-19 to elucidate patterns between disease severity and the humoral immune response.

Specific aims

Aim #1: Test sera from COVID-19 patients for SARS-CoV-2 and medically relevant HCoV-reactive antibodies. Determine the kinetics of acute and convalescent sera immunoglobulins (Ig), IgM and IgG, respectively.

Aim #2: Investigate associations between SARS-CoV-2-specific antibody titer and kinetics and disease severity in COVID-19 patients.

Aim #3 Quantify the seroprevalence of HCoVs and investigate any disease severity associations with cross-reactive SARS-CoV-2 antisera.

Significance

Viruses classified in the genus Betacoronavirus are known to cause acute respiratory disease in human populations, including the ongoing COVID-19 pandemic that, at the time of writing, has infected over 14.3 million persons globally and led to at over 600,000 deaths. Current knowledge suggests that bats are the wildlife hosts of several betacoronaviruses, implicating zoonotic spillover as the source of betacoronavirus outbreaks. Primary surveillance measures focus on nucleic acid detection of an active viral infection, but serological surveillance of these viruses is limited to a handful of emergency use authorized immunoassays. Understanding the kinetics of the human immune response, as well as antibody ability to recognize conserved and
semi-conserved epitopes across betacoronaviruses permits a controlled investigation into SARS-CoV-2 specific antibody responses and an understanding of how conserved epitopes across betacoronaviruses promote cross reactive antibodies, facilitating rational vaccine design for emerging zoonotic betacoronaviruses.

Chapter 2 – Background and Literature Review

Respiratory viruses pose a significant risk for respiratory infection and disease globally, especially in infants, the elderly, and the immunocompromised. Many viral respiratory infections present with similar clinical symptoms, highlighting the need for precise and accurate diagnostic capabilities to correctly diagnose and treat patients. Additionally, these viruses often possess the ability to spread person-to-person via droplets and aerosols, and correct information about the type of virus and mechanisms of spread can dictate the personal protective equipment (PPE) that healthcare staff are required to use during treatment and protection of the public at large. Indeed, of the four major viral pandemics of the 20th century, three were caused by respiratory viruses capable of person-to-person transmission. Understanding the pandemic potential of known respiratory viruses has been a point of emphasis, and the global health response surrounding the 2009 H1N1 outbreak shows that public health experts are learning from lessons past. Further, the recent emergence of novel coronaviruses underscores the importance of heightened bio-surveillance activities to preemptively protect global health and economic security.

In the 1960’s the first two coronaviruses, human coronavirus 229E (HCoV-229E) and human coronavirus OC43 (HCoV-OC43), were discovered when investigating the common cold. The respiratory disease associated with these viruses is mild for most of the population but
can cause more severe disease for infants, the elderly, and persons with significant comorbidities. In 2003, severe acute respiratory syndrome coronavirus (SARS-CoV) emerged in the Guangdong province of Southeastern China, resulting in ~8,000 infections and ~800 deaths\(^8\). Subsequent serum analysis from suspected SARS patients led to the discovery of human coronavirus NL63 (HCoV-NL63) and human coronavirus HKU1 (HCoV-HKU1), with disease presentation similar to HCoV-229E and HCoV-OC43\(^9,10\). In 2012, a novel viral isolate, MERS-CoV, was recovered from a patient suffering from acute pneumonia at a hospital in Jeddah, Saudi Arabia\(^11\). Since the initial discovery, MERS-CoV outbreaks continue annually, typically contained within the Middle East, but instances of travel-associated and nosocomial spread have occurred with a case-fatality rate of 34%\(^12,13\). In 2018, SARS-CoV and MERS-CoV were named by the WHO as blueprint priority diseases as representatives of the broader class for research efforts due to the public health risk stemming from their epidemic potential and lack of countermeasures\(^14\). In late 2019, cases of severe acute respiratory distress identified in Wuhan, China, led to the discovery of a new coronavirus, SARS-CoV-2, that has subsequently led to at least 14.3 million confirmed cases and over 600,000 deaths worldwide, resulting in the WHO declaration of a public health emergency of international concern due to the pandemic\(^1\).

In the United States, laboratory diagnostic measures for SARS-CoV-2 consists mainly of PCR-based assays to detect copies of the viral genome\(^2\) in the right clinical setting, but also may include serology. PCR-based assays allow for high-throughput diagnostics at a lower cost compared to other methods, but due to limited kit availability and high demand, broader screening initiatives are stalled. To better estimate the true disease burden at the time of the outbreak, efficient contact tracing and testing should be performed for all suspected and exposed cases of infection. However, although there are efforts attempting to increase such activities, this
possibility seems unlikely, as those who have been previously infected and have recovered will unlikely carry copies of the viral genome that can be detected by these PCR-based assays.

Serology-based assays, primarily enzyme-linked immunosorbent assays (ELISAs), allow for a larger window of opportunity for epidemiological studies on disease prevalence to occur, due to the prolonged nature of humoral immunity. However, these assays can be costly and labor intensive, and interpretations of these types of assays can be challenging due to antibody cross-reactivity and the variety of virus antigens used between assays. Adaptation of an ELISA-based assay onto a Luminex xMAP-based platform has previously been proposed as an alternative method of serological analysis that could address the limitations of an ELISA-based platform. In this platform, antigens targeted in the humoral immune response would be tested in multiplex for serological binding which allows for the determination of antibody cross-reactivity with more precision than with ELISA.

Initial studies of the humoral response in COVID-19 patients suggest a nearly 100% seroconversion rate by 2 weeks post-disease onset, as well as significantly improved sensitivity in clinical diagnosis when combining PCR and serological-based assays within 1 week of disease onset. However, investigations of related betacoronovirus antibodies suggest a relatively short-lived immune response along with a potential for reinfection. Additionally, preliminary evidence in SARS-CoV-2 patients suggests differential immune responses depending on disease severity, as well as an early reduction of IgG and neutralizing antibodies in the convalescent phase. A thorough understanding of the immune response can help inform rational vaccine design, and patterns in the immune response can aid in the planning of surveillance activities.

Understanding the binding and neutralizing profile of antibodies generated from SARS-CoV-2 infections can play an important role in rational vaccine design. Patterns of conserved
binding epitopes across species may elucidate the cross-neutralizing antibody response from one vaccine relevant to protection against the next coronavirus to emerge. Additionally, conserved or semi-conserved binding epitopes may elicit memory responses which can help structure cross-sectional sero-surveys around the seasonal coronaviruses to better capture the true seroprevalence of SARS-CoV-2 reactive antibodies.

**Chapter 3 – Data and Methods**

This study used a prospective, longitudinal cohort to analyze COVID-19 disease among enrollees from five military treatment facilities (MTFs) across the continental United States. The MTFs included Walter Reed National Military Medical Center (WRNMMC) (Bethesda, MD), Brooke Army Medical Center (BAMC) (San Antonio, TX), Navy Medical Center San Diego (NMCSD) (San Diego, CA), Madigan Army Medical Center (MAMC) (Tacoma, WA), and Fort Belvoir Community Hospital (FBCH) (Fort Belvoir, VA). Participants were drawn from populations that travel to or reside in different SARS-CoV-2 affected regions within the United States, including civilians and military personnel. Enrollments were without regard to age, race, and gender. Inclusion criteria included laboratory-confirmed presence of a respiratory infection at a participating MTF, were a person under investigation for a pathogen of interest (PUI) as specified by the Centers for Disease Control (CDC) guidelines, or were asymptomatic and/or had tested negative for a respiratory pathogen but were considered to have/had a recent exposure to the pathogen of interest.

The data source for the age, sex, race, military affiliation, military branch, enrollment site, smoking status, vaping status, obesity, diabetes, chronic cardiac disease, chronic pulmonary disease, hospitalization status, disease severity and sampling dates were determined from the
patient’s enrollment data via a standardized enrollment form. PCR data was generated using the Panther Fusion SARS-CoV-2 real-time (RT) PCR assay. Mean fluorescent intensity, the raw readout of the SARS-CoV-2 IgG and IgM result, and HCoV result were determined by MMIA.

The variables collected in this study included age, sex, race, military affiliation, military branch, enrollment site, smoking status, vaping status, obesity, the presence of diabetes, chronic cardiac disease, or chronic pulmonary disease, SARS-CoV-2 PCR diagnosis, hospitalization status, disease severity, date(s) sampled, microsphere-based multiplex immunoassay (MMIA) mean fluorescent intensities (MFI) for both IgG and IgM for SARS-CoV-2, SARS-1, MERS, HKU1, and OC43 spike proteins, SARS-CoV-2 IgG MMIA result, SARS-CoV-2 IgM MMIA result, and HCoV MMIA result.

Participants with missing SARS-CoV-2 diagnostic tests were removed from the data set, and remaining participants were grouped into two groups based upon PCR result. Overall demographic summaries for the categorical variables were constructed. Continuous variables from each group were used to generate boxplots. These variables were tested for independence employing the Chi-Squared Test for Independence and the Fisher’s Exact Test to elucidate potential interactions between categorical variables and PCR result. The PCR positive group was further tested employing the Chi-Squared Test for Independence and the Fisher’s Exact Test to incorporate interactions with disease severity, characterized by hospitalization status, limitation of physical activity, and the requirement and type of therapeutic oxygen. Finally, the SARS-CoV-2 IgG titer was investigated in relation to disease severity using both an Independent Samples T-Test and an ANOVA.

Generalized Estimating Equations were performed on SARS-CoV-2 IgG and IgM MFI values in order to detect seroconversion as well as evidence of waning immunity. The mean days
post symptom onset for PCR positive, IgG positive, IgM subgroups was analyzed using an Independent Samples T-test in order to detect a difference in means between the two groups. Finally, the IgG MFI values for each of the coronavirus antigens were plotted in order to find patterns of reactivity throughout the time-course.

Chapter 4 – Results

Study population

Summaries of participant information grouped by PCR status can be found in Table 1. The study population was diverse: the average age of study participant was 45.8 years with a standard deviation of 15.8, a minimum age of 13.5 and a maximum age of 84.5. Twenty-two participants were Black, fifty-one White, twenty-eight Hispanic, five native Hawaiian, nine Others, and one had a missing value. There were sixty-six male and fifty female participants. Thirty-nine participants were active duty members of the military, thirty-six were retired military, thirty-nine were military dependents, one was a civilian and one had a missing value. Of the military associates, fifty-four were affiliated with the Army, twenty-nine Navy, nine Marines, twenty Air Force, one Coast Guard, one listed as other, and one had a missing value. Seventy-two participants had a recorded SARS-CoV-2 PCR positive result, while thirty-four were recorded as negative and ten results were missing. Sixty-six participants were treated as outpatients, forty-three were inpatient, and seven had missing values. Of all of the participants, thirty-four were listed as outpatient without limited physical activity, thirty-two were listed as outpatient with limited physical activity, sixteen as inpatient without the requirement of supplemental oxygen, twenty-three as inpatient with non-invasive oxygen required, four as inpatient requiring ventilation, and seven with missing severity levels. Six of the participants
were active smokers, thirty-three were former smokers, seventy-five never smoked, and two had missing values. Two participants were active vapers, seven had previously vaped, one-hundred-four had never vaped, and three had missing values. Twenty-two participants were obese, while ninety-three participants were not, and one had a missing value. One-hundred-two participants were not diabetic, thirteen were, and one was missing. One-hundred-six participants reported no chronic cardiac disease, while nine did with one missing. One-hundred-ten participants reported no chronic cardiac disease, while five did with one missing. The average collection date of samples post symptom onset of was 33.5 days with a standard deviation of 19.0, a minimum of one, and a maximum of one-hundred-three. Boxplots showing the distributions of age and days post symptom onset at the time of sampling can be found in Figure 1.
<table>
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<tr>
<th>Variable Category</th>
<th>PCR Negative (n=34)</th>
<th>PCR Positive (n=72)</th>
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<td></td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
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<td><strong>Age Group</strong></td>
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<td>10 to 20</td>
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</tr>
<tr>
<td>20 to 30</td>
<td>9 (8.5)</td>
<td>14 (13.2)</td>
</tr>
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<td>30 to 40</td>
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<td>3 (2.8)</td>
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<td>25 (25.6)</td>
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<td>50 to 60</td>
<td>7 (6.6)</td>
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<td>60 to 70</td>
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<td>15 (15.6)</td>
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<td>70 to 80</td>
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<td>80 to 90</td>
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<td>Female</td>
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<td>FBCH</td>
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<td>Outpatient, lethal activity</td>
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<td>19 (17.5)</td>
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<td><strong>Chronic Pulmonary Disease</strong></td>
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<tr>
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<td>1 (0.9)</td>
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Table 1. Categorical Variables of Study Participants separated into PCR negative and PCR positive groups.
Figure 1. Boxplots of the continuous variables separated by PCR status. A) Distribution of Age and B) days post symptom onset at the time of sampling.
Chi-Squared Tests for Independence and Fisher’s Exact Tests were used to determine any associations between categorical variables (Table 2). For the entire population, disease diagnosis was associated with the site of enrollment (p<0.0001), due to the WRNMMC facility only enrolling PCR positive patients. Among the PCR positive participants disease severity was associated with race (p=0.01), military affiliation (p=0.0005), site of enrollment (p<0.0001), chronic cardiac disease (p=0.038), age group (p=0.0372), and obesity (p=0.012), and chronic cardiac disease was associated with chronic pulmonary disease (p=0.0136). As expected, military affiliation was confounded by age, with older participants primarily grouped into the retired military subset.

<table>
<thead>
<tr>
<th>Variables of Significant Association</th>
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<tr>
<td><strong>Diagnosis</strong></td>
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<td>Site</td>
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<tr>
<td><strong>Severity</strong></td>
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<tr>
<td>Race</td>
</tr>
<tr>
<td>Affiliation</td>
</tr>
<tr>
<td>Site</td>
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<td>Chronic Cardiac Disease</td>
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<tr>
<td>Age Group</td>
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<td>Obesity</td>
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<tr>
<td><strong>Chronic Cardiac Disease</strong></td>
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<td><strong>Chronic Pulmonary Disease</strong></td>
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<table>
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<th>Variables</th>
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<th>Test</th>
<th>Significance</th>
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</thead>
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<td>Fisher’s Exact Test</td>
<td>p&lt;0.0001</td>
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<tr>
<td>Severity</td>
<td>72</td>
<td>Fisher’s Exact Test</td>
<td>p=0.01</td>
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<td>Race</td>
<td>72</td>
<td>Fisher’s Exact Test</td>
<td>p=0.0005</td>
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<td>Affiliation</td>
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<tr>
<td>Site</td>
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<td>Chronic Cardiac Disease</td>
<td>72</td>
<td>Fisher’s Exact Test</td>
<td>p=0.0136</td>
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</table>

**Table 2. Categorical variables with significant association.** A two-tailed Chi-Squared Test for Independence was used to detect associations between categorical variables. Fisher’s Exact Tests were used when the Chi-Squared assumptions were not met.
Main results of Antibody Testing

To investigate patterns of Ig decay, two methods were used. First, the difference in days post symptom onset for 20 IgM negative and 95 IgM positive samples collected in PCR positive, and IgG positive participants was investigated using an Independent Samples T-Test. Equal variance was observed so a Pooled analysis was used. The mean difference was 7.15 days greater in IgM negative samples (p=0.0368), indicating significant IgM decay relative to IgG. Next, a Generalized Estimate Equation was used to predict the factor of IgM decay over time. Significant IgM decay was detected (p=0.0007), with a predicted loss of 130 MFI per day. A Generalized Estimate Equation was again used to determine IgG decay over time. Although the equation predicted IgG decay over time, the factor was determined to be non-significant (p=0.09).

<table>
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<th>Difference in Days Post Symptom Onset</th>
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<td><strong>Mean Difference</strong></td>
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<td>7.15</td>
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</table>

**Table 3. Statistical Analysis of Immune Kinetics.** A) Independent Samples T-Test to detect differences in mean days post symptom onset between IgM negative and IgM positive groups. B) Generalized Estimate Equation to detect decay in IgM titer (measured with MFI) over time. C) Generalized Estimate Equation to detect decay in IgG titer (measured with MFI) over time.
Due to limitations in the samples collected, investigations into the timing of seroconversion were not possible. However, trends in the time-course could be analyzed for two of the three relevant participants (Figure 2). In both persons, an atypical immune response involving IgG and IgM peaking together was observed at or shortly after ten days. More investigation into the kinetics of other participants is required to determine any relevant patterns.
Figure 2. Graphs of immune kinetics. Time-course graphs for participants S00-0014 (top) and S00-0022 (bottom). Mean fluorescent intensity is used as a measure of Ig titer. In both graphs, IgG is represented in red, while IgM is in blue.
Two methods were used to investigate differences in SARS-CoV-2 IgG magnitudes with disease severity (Table 4). First, an Independent Samples T-Test was used to compare the mean MFI values between in- and outpatient participants at least 28 days post symptom onset. Unequal variance was observed between the two groups, so a Satterthwaite analysis was used. The mean MFI value for the hospitalized group was 11048.8 units higher than in the outpatient group, indicating a significantly higher IgG magnitude (p<0.0001). Next, a one-way ANOVA was used to compare the mean MFIs between severity categories. Again, significant differences were observed (p<0.0001). Unsurprisingly, the more severe groups (requiring inpatient treatment) had higher MFI values than did the less severe groups, however there were no observed differences within the outpatient or inpatient severity subsets.

<table>
<thead>
<tr>
<th>Mean Difference</th>
<th>Standard Error</th>
<th>Degrees of Freedom</th>
<th>t Value</th>
<th>Significance</th>
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<tbody>
<tr>
<td>11048.8</td>
<td>1762.1</td>
<td>99.8</td>
<td>7.47</td>
<td>&lt;0.0001</td>
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</table>

Table 4. Statistical analysis of SARS-2 IgG magnitude by disease severity groups. (A) Pooled, independent samples T-Test for differences in SARS-CoV-2 IgG MFI by hospitalization status. (B) One-way ANOVA comparing differences in SARS-CoV-2 IgG MFI by disease severity.
Other results

Investigations into the impact of prior HCoV exposure on disease severity were not possible due to an extremely high HCoV IgG prevalence in the study population. However, interesting patterns from the two time-course patients were observed. In both participants, OC43-reactive antibodies are detected at the first time point in relatively high-titers and climb throughout the time-course. Additionally, reactive antibodies to SARS-1 and MERS climb in nearly identical patterns that follow the growth of SARS-CoV-2 specific antibodies.
Figure 3. Graphs of IgG kinetics across viral species. Time-course graphs for participants S00-0014 (top) and S00-0022 (bottom). Mean fluorescent intensity is used as a measure of IgG titer. In both graphs, SARS-CoV-2 is represented in red, SARS-1 in olive, MERS in charcoal, HKU1 in green, OC43 in blue, and mock in purple.
Chapter 5 – Discussion

In this project, I sought to investigate relevant demographic and clinical characteristics in a prospective cohort of patients enrolled at MTFs and their association with COVID-19 disease severity, as well as the important humoral immune characteristics as they relate to COVID-19. Unsurprisingly, many of the characteristics, such as age, race, obesity, and chronic cardiac disease, were confirmed to be associated with disease severity as reported elsewhere\textsuperscript{22-26}.

Significant trends were discovered in the immune responses of this cohort that may inform public health practice. First, canonical IgM decay was observed, whereas a non-canonical primary Ig response occurred where IgM and IgG titers rose simultaneously. This pattern, along with high levels of OC43 IgG that rose alongside SARS-CoV-2-specific IgG, indicates a possible anamnestic response triggered by SARS-CoV-2 exposure may be accelerating the temporal detection of SARS-CoV-2 reactive IgG. However, limitations in sample size do not allow for a determination of significance. Additionally, a higher magnitude IgG response was detected in patients with more severe disease. Recent studies have found a similar trend and suggested that the association between severe COVID-19 coincided with viral infections of the lower respiratory tract, which were hypothesized to require higher levels of antibody in order to reach infected lung tissue\textsuperscript{27}. Together, this information suggests that the elderly and persons with significant comorbidities should be prioritized for measures designed to limit SARS-CoV-2 exposure to minimize the number of severe COVID-19 cases.

This project had a few limitations that are important to discuss. Most importantly, more samples from more participants would allow for a more thorough understanding of the associations above. Trends from the two time-course participants may be highlighted or muted with more time-course participants. Additionally, the DoD-eligible and DoD-beneficiary
population in this study might not be reflective of the overall United States population. Specifically, the male to female ratio is inconsistent with the US population, and smokers and persons with significant comorbidities were underrepresented in the cohort. However, some trends found in the general population were also found within this cohort, leading me to believe that this study can be generalizable across the US.

These results raise more questions about public health policy moving forward. Prior viral exposure and disease severity should be considered when initiating a vaccine campaign. Currently, there is no evidence suggesting a low magnitude antibody response provides protective immunity\textsuperscript{28}. The neutralizing ability of these sera will need to be investigated prior to vaccination determinations. Additionally, evidence of an anamnestic response to other coronaviruses contradicts the current dogma surrounding the short-lived nature of coronavirus immunity. Investigations into the memory response generated by COVID-19 survivors will play a pivotal role in informing population vaccine planning. Finally, future sero-surveillance projects should consider the anamnestic response when planning sampling efforts. Persons who had less-severe SARS-CoV-2 infections may have lower-than-detectable levels of circulating antibody that may be increased when exposed to seasonal coronaviruses. Sampling measures that prioritize sampling during cold seasons may produce results that are more reflective of the true seroprevalence in any given population.
Cited Literature


Acknowledgments

Capstone Committee

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- Sharon Medcalf, PhD

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Co-curricular Activities
Undergraduate Activities:
08/11-05/12 Varsity Letter recipient and Championship Team qualifier, University of Maryland, College Park Men’s Swimming and Diving Team
08/12-05/15 Member, Students Helping Honduras
Post-graduate Activities:
06/15- Participant and presenter, Uniformed Services University Virology Journal and Data Club
06/15- Uniformed Services University Emerging Infectious Diseases seminar series

Present Duties
- Clone, express, and purify soluble envelope glycoproteins from virus species in the Filoviridae and Paramyxoviridae families
- Selection and purification of polyclonal IgG from immunized rabbit sera
- Preparation of Bio-Rad Bio-Plex beads for immunoassays
- Generation of standard curves for analysis of Bio-plex assays
- Sterile cell culture of human and bat immortalized cell lines
- Culture and experimentation with Australian bat lyssavirus to analyze the innate cellular anti-viral responses in bat and human cell lines
- Expression and purification of human and mouse monoclonal antibodies
- Immunization and production of mouse hybridomas for the expression of monoclonal antibodies
• Production and expression of human fragment antibodies (Fabs) using soluble glycoprotein and virus-like particles with bacteriophage-phage display technology
• Development of neutralization assays to assess the neutralization and cross-neutralization capacities of sera samples
• Generation of publication-worth figures using SAS and GraphPad software.
• Manuscript preparation
• Oversee and direct logistics for international collaborations

Publications


Yan L., Sterling S.L., Laing E.D., and Broder C.C. Expression System for Recombinant Henipavirus Glycoproteins. *(submitted).*


**Research Posters Presentations**
