Spring 4-12-2021

Development of an Immunoblot to Detect Human Antibodies Against SARS-COV-2 Virus Proteins

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DEVELOPMENT OF AN IMMUNOBLOT TO DETECT HUMAN ANTIBODIES AGAINST SARS-COV-2 VIRUS PROTEINS

By
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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College

Oxford
May 2021

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DEDICATION

I would like to dedicate this capstone project to my parents, Sanjay and Karuna Desai. I am extremely grateful to have parents that always support and encourage me in all my endeavors. Because of them, I have had the opportunity to put forth my time and effort into my senior thesis for the Sally McDonnell Barksdale Honors College.

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Wayne L. Gray and The University of Mississippi Biology Department for the opportunity to participate in this research project. Dr. Gray's determination and innovation has expanded my knowledge in the world of virology and helped me pursue my goals in science.

I also would like to thank the Honors College for giving me this opportunity to culminate my time into a project that will supplement my academic career.

ABSTRACT

SHIVUM KUMAR DESAI: Development of an Immunoblot to Detect Human Antibodies Against SARS-CoV-2 Virus Proteins

(Under the direction of Dr. Wayne L. Gray)

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is a virus that infects cells in the lungs, nasal passages, and intestines via the ACE2 receptors of the host, and leads to the coronavirus disease (COVID-19). Since the occurrence of the pandemic in December 2019, there have been 114 million cases worldwide in which 2.5 million lives have unfortunately been taken away.

Being diagnosed with a past infection stems questions among those that have tested positive through antibody testing. The problem here is that patients were notified that they have antibodies resulting from a SARS-CoV-2 infection, but in fact they may not possess these antibodies which lead to a false positive result. This is detrimental, as patients may believe they are immune to the virus due to a perception of acquired immunity. Patients will then go out in public at free will, which can result in the contraction and further transmission of the virus.

This thesis follows the goal to develop a protein purification protocol and an immunoblot technique that can successfully identify the presence of human antibodies in response to the nucleocapsid (N), spike (S), and receptor binding domain (RBD) proteins of the SARS-CoV-2 virus. The immunoblots, in theory, will help towards the goal of eliminating false positives that arise from other antibody tests.

To achieve the goal, the N, S, and RBD proteins were subjected to purification. *E. coli* cells were transformed with DNA genetic material that coded the proteins. The proteins were expressed, a cell lysate was formed, and the proteins were then purified through his-tag affinity column

chromatography. The purified proteins were subjected to SDS page gel electrophoresis and membrane transfer. The transferred proteins were used in a series of immunoblots with specific rabbit sera to confirm protein purification. Sera from humans were used to detect COVID-19 through antibody interactions with the purified proteins.

Results showed that human antibodies from COVID-19 patients were able to bind to the N and S protein, but not the RBD. Therefore, with more human sera immunoblot data, an immunoblot protocol can then be put into effect to diagnose patients for past infections.

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LIST OF ABBREVIATIONS

˚C	Degrees Celsius
ACE2	Angiotensin Converting Enzyme 2
CCL2	Chemokine Ligand 2
CCL3	Chemokine Ligand 3
COVID-19	Coronavirus Disease
DNA	Deoxyribonucleic Acid
E	Envelope
ELISA	Enzyme-Linked Immunosorbent Assay
FaB	Fragment Antigen Binding
Fc	Fragment crystallizable Region
G	G force
GCSF1	Granulocyte Colony Stimulating Factor 1
His-tag	Histidine Tag
HRP	Horse Radish Peroxidase
PVDF	Hydrophilic polyvinylidene fluoride
PTG	Isopropyl β-D-1-thiogalactopyranoside
IFN	Interferon
IgG	Immunoglobin G
IP10	Interferon gamma-induced protein 10
IL-2	Interleukin 2
IL-7	Interleukin 7
kb	Kilobase

kDA	Kilodalton
LB	Luria Broth
mA	Milliamp
mM	Millimolar
M	Membrane
MCP1	Monocyte Chemoattractant Protein 1
MERS	Middle Eastern Respiratory Syndrome
MW	Molecular Weight
μL	Microliter
mL	Milliliter
mRNA	Messenger Ribonucleic Acid
ng/mL	Nanogram/Milliliter
N	Nucleocapsid
Ni2+	Nickel
ORFA1a	Open Reading Frame 1a
ORFA1ab	Open Reading Frame 1ab
pp1a	Polyprotein 1A
pp1ab	Polyprotein 1AB
R	Receptor Binding Domain
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
S1	Subunit 1
S2	Subunit 2

S	Spike
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SARS	Severe Acute Respiratory Syndrome
SOB	Super Optimal Broth
TNFα	Tumor Necrosis Factor
V	Voltage

Introduction

In the world of virology, the breakthrough was the development of vaccines in the 18th Century by Edward Jenner, which foreshadowed the eradication of smallpox. Since then, numerous vaccines have been created to significantly lower the rate of infection among the human population in a plethora of viral diseases. Since the cultivation of the first vaccine, scientific discoveries have been able to give us an explanation on why our bodies are able to develop immunity. Antibodies are a key to fighting past infections. With this knowledge, many tests have been developed to identify individuals that have antibodies that are able to interact with viruses. One prominent method is the enzyme-linked immunosorbent assay (ELISA). ELISA is used to test patients that may have been previously infected with Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). The only issue that resides with this is that ELISA assays may sometimes provide false positives results. To combat this, the use of immunoblots can be used to eliminate false positive results that could arise from the ELISA assay, and a clear indication can be obtained on whether an individual has antibodies.

This project is being conducted to further research in the new field of coronavirus disease (COVID-19) diagnosis using immunoblot technology. It is hypothesized that by using the nucleocapsid (N), spike (S), or the Receptor Binding Domain (RBD) proteins in an immunoblot, patients that are COVID-19 positive should have antibodies that will bind to these proteins from the exposure, thus confirming that these patients have had the disease. The two primary goals of this project are listed as follows:

- To create an optimal protocol for purification of N, S, and RBD proteins, and their transfer in usage for immunoblots.
- To evaluate the antibody response of COVID-19 patients against the N, S, and RBD proteins.

Chapter I: Background on COVID-19

Coronavirus disease 2019 (COVID-19), as the name suggests, was first described in 2019 when the population in Wuhan, China began to experience an unknown disease outbreak. Those that were infected began to experience cases of pneumonia, which was unexpected due to thousands of people randomly having the same symptoms. However, on January 7, 2020 the World Health Organization identified the causation of this outbreak by a new coronavirus, SARS-CoV-2. Demographically, all age groups were infected, however the elderly and those that already had underlying health problems had the highest mortality rate. In March 2020, the World Health Organization declared a global pandemic as almost half of the world's countries had cases of the virus being transmitted among their respective populations.

As of 2021, almost 114 million cases have appeared over the course of the pandemic, and cases are still being reported to this day. Fortunately, 64.1 million cases have recovered, while the rest are currently undergoing treatment. There have been 2.5 million deaths worldwide giving the mortality rate of 1.4% (World Health Organization, 2021). In the United States, 30 million cases have arisen, and 543 thousand deaths have occurred. From the beginning of the pandemic to now, great progress has been made within a matter of short time. Reported cases have been decreasing due to the implementation of quarantine and protective measures such as face masks in public places, and most importantly the synthesis of vaccines that have become available to the public. Furthermore, the rapid testing and availability of testing centers have made diagnosis efficient in combating transmission of the virus. The highest reported cases were seen on December 20, 2020 at 843,500 cases. However, on February 20, 2021 the number of reported cases had dropped by

nearly 50% to 398,329 (World Health Organization, 2021). This significant decrease indicates that the scientific community is moving in the right direction to slowly stop the spread of the virus.

Emergence of SARS-CoV-2

SARS-CoV-2 comes from the family *Coronaviridae*. In the past outbreaks of viruses from this family, the common factor that unites the origin are that they are zoonotic in nature (Anila, Ganesh, Jayesh, Krishnadas & Raghuvir, 2020). Some notable examples are the Middle Eastern Respiratory Syndrome (MERS), and the Severe Acute Respiratory Syndrome (SARS) which were both to be caused by transmission from animals to humans. MERS and SARS were both transmitted via bats where MERS had the dromedary camel as an intermediate host. When SARS-CoV-2 was discovered, the main goal of the scientific community was to figure out where this virus originated from.

SARS attacks the host cells via the ciliated bronchial epithelial cells by angiotensin converting enzyme 2 (ACE2). Furthermore, SARS-CoV-2 infects the host's cells in the same manner. SARS and SARS-CoV-2 are genetically different, but according to homology modeling, both viruses have similar receptor binding domain structures (Anila, Ganesh, Jayesh, Krishnadas & Raghuvir, 2020). Though to this day, the origin of SARS-CoV-2 is still scientifically unknown, although hypotheses are suggesting that due to the similarities between SARS-CoV-2 and SARS, bats may also be the origin.

Animal to human transmission can occur randomly. The nature of viruses is constantly changing due to genetic drift. Climate change and cross contamination between humans and animals are increasingly more prevalent, especially in China where the epicenter of the pandemic was located. The nature of SARS-CoV-2 being an RNA virus is shown to be more susceptible to

mutations, leading different strains to arise. In the United Kingdom, a strain known as B.1.1.7 emerged in December 2020 and became the most prevalent strain in the country (Bollinger & Ray 2020). Another strain that is prevalent in Brazil, South Africa, and some parts of California is B.1.351. Both strains could have mutations on spike proteins which are located on the surface of the virus. This could allow for better infectivity and result in quicker transmission across a population (Bollinger & Ray 2020). However, not enough research has been conducted on whether this hypothesis is true. Furthermore, the rise of different strains of COVID-19 can lead to complications with the current vaccines that are available to the public. This may lead to the emergence of new vaccines, just like the seasonal flu.

Morphology, Virus Structure, and Replication

The virus's structure is important to understand in which logical sense can then be made on how the virus enters the host's cell. A mature SARS-CoV-2 particle is spherical and enveloped. The virus contains a single stranded positive sense RNA as the genetic material in which the size of it is 26 to 32 kb (Bergmann & Silverman, 2020). The genome codes for four main proteins the virus utilizes for replication. The four main proteins are the spike protein (S), the envelope protein (E), the membrane protein (M), and the nucleocapsid protein (N). Figure 1 shows the location of each protein in relative to each other. The S protein, located on the membrane, consists of trimeric glycoproteins which aid in host attachment. Located in the S protein is the receptor-binding domain (RBD) protein which allows the S protein to complete its function of entry to the host cell. The N protein is in the nucleocapsid and consists of two domains that both utilize different mechanisms to bind to RNA. The E protein is a transmembrane protein that facilitates the assembly of the virus and allows for the release. Further research is still being conducted on this specific protein. The M

protein is small in nature containing three transmembrane domains giving rise to the viral spherical structure.

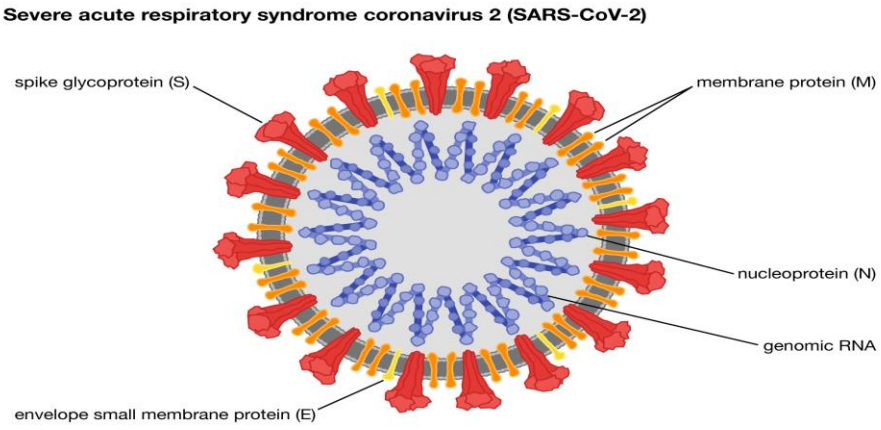

Figure 1. Structure of SARS-CoV-2 and the prominent proteins. Throughout the study, the three main proteins used were the N protein, the S protein, and the RBD protein located in the S protein.

Binding of the virus to the host cell requires proteases and the two subunits of the S protein (S1 and S2) (Bergmann & Silverman, 2020). The S1 subunit contains the RBD protein which binds to the angiotensin-converting enzyme 2 (ACE2) receptor of the host cell. The S2 subunit is cleaved within two sites by a serine protease. The first cleavage separates the S1 from the S2 site, and the second cleavage allows for the exposure of fusion peptides. The fusion peptide inserts into the host membrane which then creates an antiparallel six-helix bundle (Bergmann & Silverman, 2020). Both membranes can now fuse, and the bundle then allows for the genetic information from the virus to be inserted into the host's cytoplasm. The viral proteins are then synthesized using the host's ribosomes. The polyproteins pp1a and pp1ab, that are newly synthesized by open reading frame 1a (ORFA1a) and ORFA1ab, are processed by viral proteases which then make up nonstructural proteins that are sent to the Golgi apparatus where the four main proteins are created (Bergmann & Silverman, 2020). Positive sense RNA is created in the host's nucleus by RNA

transcriptase. Then these newly assembled viral particles are released via exocytosis. Viral infection is completed, and the lytic life cycle for the virus repeats.

Viral Transmission

SARS-CoV-2 spreads between individuals via droplet transmission, airborne transmission, and contaminated surfaces. Most of the viral transmissions take place on mucous membranes, primarily the ones that are lined across the nose and mouth (Center for Disease Control and Prevention, 2020). Eventually, infection leads to an upper respiratory tract infection, as well as a lower respiratory tract infection. Droplets in the air eventually decrease in size in relation to the distance from the infected individual. While the droplets are in the air, non-infected individuals can inhale these droplets resulting in airborne transmission. Evidence shows that in enclosed spaces and inadequate ventilation can lead to spread of the disease (Center for Disease Control and Prevention, 2020). However, close person to person contact is more effective than airborne transmission. Contaminated surfaces with droplets of concentrated amounts of the virus can cause infection. If an individual touches these contaminated surfaces and then touches their own nose, eyes or mouth, the individual can infect themselves. These methods of transmission are expressed from hosts that are symptomatic and uniquely asymptomatic as well.

Not only human to human transmission is observed, but concern of animals transmitting the virus to humans has been investigated. An infected tiger in a New York zoo raised alarms that domesticated cats could cause transmission inter-specially (Center for Disease Control and Prevention, 2020). Although, both cats and ferrets have been observed to transmit intra-specially, there is little evidence that humans can get infected from domesticated animals.

Illness and symptoms

SARS-CoV-2 produces a wide spectrum of symptoms for each individual patient. Some patients can experience symptoms such as flu-like, fever, dry cough, and fatigue. Other patients can be asymptomatic and feel completely fine but are still contagious. Few people can have adverse symptoms such as acute respiratory distress syndrome and an uncontrolled systemic inflammatory response called a cytokine storm that leads to organ failure and death (Yanwen & Lien 2020). Symptoms appear within two to fourteen days for patients and therefore can last almost two weeks before becoming noncontagious. Those with critical symptoms appear to recover within three to six weeks. Multiple patient accounts have also testified the loss of smell and taste after recovery from the initial infection. About 5-10% of patients still have anosmia and ageusia after for an additional four-six weeks (Zhang & et al 2020).

Once infected however, the immune system produces type 1 interferon (IFN) and proinflammatory cytokine responses to combat the virus at an early stage. Other cytokines that have been found in high concentrations in response to SARS-CoV-2 are IP10, GCSF1, MCP1, and TNFα (Bergmann & Silverman, 2020). However, the result of too many cytokines lead to the effect of a cytokine storm, which is the predominant reason for organ failure and death. An abundance of interleukin (IL)-2, IL-7, GCSF1, IP10, CCL2, CCL3, and TNF-α have been seen in patients that ultimately die. However, more research is needed to confirm this hypothesis of the cytokine storm and why the hyperinflation of cytokines are seen in patients that experience complete failure of organs.

Patients that have unfortunately been deceased had shown low levels of CD4+ and CD8+ T-cells. It is hypothesized, due to lack of research, that lymphocytopenia occurs through inducing

T-cell apoptosis or autophagic cell death (Bergmann & Silverman, 2020). SARS-CoV-2 could infect T-cells via S mediated membrane fusion.

What makes asymptomatic patients able to evade the symptoms the virus induces? Currently, it is unknown due to lack of studies on these specific groups of people. However, different ACE2 expressions could be a factor within each individual case. But future research via antibody testing and other means could give more insight.

Diagnosis

Being tested is extremely important in the combat of transmission. Individuals that are in constant contact with others or in crowded environments should get tested to diagnose correctly and take affirmative action. Currently, the main detection for SARS-CoV- 2 is reverse transcriptase polymerase chain reaction (RT-PCR) (Zhang & Le, 2020). Patient samples are collected by nasopharyngeal swabs or oropharyngeal swabs. The reverse transcriptase reaction then converts RNA into DNA and amplification of the DNA is induced through multiple cycles. Fluorescence is then used to detect the DNA if present by cleaving the 5' exonuclease activity and then removing the quencher from the fluorophore (Zhang & Le, 2020). Thus RT-PCR can be used to diagnose patients that may have active infections. Another form of diagnosis for active infection is rapid antigen tests. These tests measure the antigens of the virus in the patient. A positive result happens when the antigens that are obtained from a patient through nasopharyngeal swaps bind to the antibodies that are already bound onto the test. A colored line will be generated once the antibodies and antigens react. The issue with rapid antigen testing is that there needs to be a high virus cell count so antigens can be recognized. Therefore, the limitation of this test is that it is most reliable

when the patient is in the early active infection stage due to the high cell count of the virus in the nose and throat.

Antibodies to viral proteins can be used to confirm past infection diagnosis. Currently the enzyme-linked immunosorbent assay (ELISA) is used for the detection of antibodies against the S protein. However, ELISA can generate false positive results due to the low antibody levels or from laboratory error.

Immunoblots have much higher sensitivity and can therefore eliminate possibilities of a false positive and produce a more accurate indication of the presence of antibodies. A positive result from an immunoblot is only seen when the band that is produced on a membrane is the correct size. Therefore, immunoblots are more reliable than ELISA, as a size of the protein in which the antibodies bind to is considerable.

Treatment and Prevention

Current treatments

There were at least three potential therapeutic methods against SARS-CoV- 2. The first being direct antiviral drugs such as remdesivir (Fehr & Perlman, 2015). The antiviral drug works by inhibiting one of the key enzymes used for viral replication of RNA. In turn, viral nucleic acid multiplication is inhibited. The second therapeutic method consists of injection of convalescent plasma that has antibodies against SARS-CoV-2. The antibodies that are produced by a patient that has successfully recovered from infection are used to treat infection within another patient. The third method that targets pathogenicity of the virus is the use of dexamethasone which reduces the pathogenic hyperactive inflammatory response that can create the cytokine storm in disease

progression. By repressing certain cytokines, hyperinflammation is reduced and the potential for organ failure is ceased.

Preventative measures

The most impressive scientific accomplishment that has happened within the past year is the rapid production of vaccines that use a different mechanism of action in comparison to older vaccines for other viral diseases. Respectively, PfizerBioNtech and Moderna both utilize mRNA as the premise of the vaccine rather than using traditional conventions of attenuated viral particles. The mRNA inside the vaccines code for the spike protein using the host's cells machinery. Once the surface proteins are created, the spike proteins are presented (Center for Disease Control and Prevention, 2020). A normal immune response is activated and after removal of all the proteins, memory cells are created in response for future infections from the live SARS-CoV- 2 virus itself. Due to the nature of SARS-CoV- 2 being able to mutate, different strains may cause complications for the efficacy of the vaccine. However, due to the nature of the vaccine being primarily mRNA based, the genetic code can be changed according to the strain, and booster shots could be administered to combat the constant mutation of the virus. Since the public dispatch of the vaccines, reported cases are at declining levels. Despite the positives of the vaccine, there has been a lot of opposition to it due to the hysteria of it not being completely safe. Many figures, including the President of the United States, have taken the vaccine to showcase the effectiveness and safety of this scientific advancement.

Multiple rules and regulations have been put in place to halt the spread of SARS-CoV- 2. Masks are required to be worn in all public places to prevent the transmission of respiratory droplets. Multiple lockdown methods across nations have been utilized to prevent social mass

gatherings and thus spreading the disease even more. If an individual has met a patient that has been tested positive, self-isolation methods have been made aware to the public to fight against the spread of the disease. Finally, practicing safe measures such as washing hands, staying hydrated, sanitizing, avoiding indoor unventilated areas, staying 6 feet away from others and covering coughs and sneezes are all ways to limit the transmission of SARS-CoV- 2 significantly.

Research Model and Limitations

The goal of this project is to create an effective technique that can identify the presence of antibodies from human serum by exposing them to purified proteins that are associated with SARS-CoV- 2. The method that this project can be conducted by is through the technology of immunoblotting. Studies first must be conducted on rabbit sera to show the effectiveness of the immunoblot and the presence of the viral proteins. Once desired results are obtained, human serum can then be tested to see the effectiveness of the model, and then determine the steps that need to be taken. The proteins that are used in the immunoblot must be chosen under careful consideration to make sure the specific antibodies that are being recognized bind to the protein just like a real-life infection to indicate the presence of the viral structures.

There are a total four main structural proteins that have been identified in SARS-CoV- 2. The one that is most used for treatments is the spike protein due to the accessibility and function of it. Being a surface bound protein makes it an easy target for antibodies to recognize. In addition, the receptor binding domain protein is in the spike 1 subunit of the structure. Utilizing a response to this protein will hinder the effects of attachment that is necessary for the lifecycle of the viral particle. Thus, using it in an immunoblot would serve the purpose of identification via useful means. Another good candidate for the experiment would be the nucleocapsid protein. Even

though the location of the protein is found in the nucleocapsid, hence the name, the structure itself is heavily phosphorylated (Bergmann & Silverman, 2020). Just like the spike and receptor binding domain protein, utilizing the nucleocapsid protein in the experiment would lead the identification by antibodies from sera useful.

The study in this thesis is based on the research conducted in the past year. False positives from enzyme linked immunosorbent assays (ELISA) can be a limitation in the field of identification of the virus. To reinforce the result, immunoblotting would work effectively due to the high sensitivity to specific binding. Immunoblotting also does have its limitations. The time-consuming nature of this procedure is a drawback since results for patients would be more effective when displayed quicker. This limitation also roles over to the fact that antibodies can also stay in patients for periods of time. Thus, a positive result from an immunoblot may not give the result of an active infection. However, this can be advantageous as patients may not have realized they have built the immunity to the disease due to the asymptomatic nature that patients see. Despite the limitations, the modes for this experiment to identify will parallel with the aims of prevention of COVID-19, and ultimately provide a pathway for an improvement in the future.

CHAPTER II: METHODS

The study in this thesis is approached by basic scientific methods to both purify and transfer proteins in which detection can then be employed through the means of immunoblotting. Purifying the protein involved transferring the genetic material that synthesizes the SARS-CoV-2 proteins into BL21 *E. coli* strained cells. The genetic material that was transferred is made by inserting the base pair sequence that codes for the SARS-CoV-2 N, S, and RBD proteins inside a vector of the *E. coli* cell. The vector that was used was a plasmid inside the bacteria. As well as the base pair sequence, six histidine tags were attached to the protein so the protein purification could be conducted based on affinity later in the protocol. Figure 2 shows a visual perspective of how the SARS-CoV-2 DNA sequence and histidine tags were created.

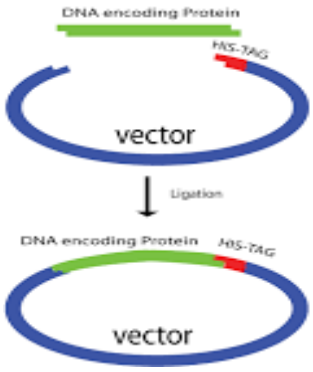

Figure 2. Basic premise of how the plasmid in the thesis was made. DNA encoding the SARS-CoV-2 N, S, RBD with a his-tag was inserted into a plasmid vector used and used to transform the *E.coli* cells.

Expression of the product by inoculation and induction of proteins was carried out after, followed by lysing the cells to isolate the proteins in preparation for the purification steps. Once the protein had been obtained, different gels were conducted to determine the optimal amount of protein needed so created stock could be used sparingly. Purified proteins were then transferred onto a PVDF membrane, which were then cut up and soaked in irradiated sera. This initiated binding of antibodies to the purified proteins. The process of immunoblotting required both primary and secondary antibodies to detect the data of interest. Chemiluminescence was used to identify bands on the PVDF membrane in correlation with the specific protein-antibody interactions. Figures were collected and interpreted to assess the results from the experiments afterwards.

Sera, obtained from RayBiotech and Sino Biological were drawn from the respected animals and humans, frozen down and shipped to the University of Mississippi for experimentation. Careful handling with gloves under the fume hood was practiced ensuring safe procedures were carried out. Furthermore, the sera used in this thesis were both employed as positive and negative controls meaning that antibodies were either present or absent in accordance with the purified proteins. The sera had also been tested by the means of enzyme linked immunosorbent assay (ELISA). So, immunoblotting the sera truly meant to test false positives that may arise from ELISA assays. Furthermore, human sera were obtained from multiple patients to create accurate and reliable results. Each serum was then exposed to nucleocapsid protein, spike protein, and receptor binding domain protein to assess the antibody interactions. From here, membranes were disposed of and results were recorded properly.

Protein Purification Protocol

The protocol began by the transformation BL21 strained *E. coli* cells. 1μL of protein encoding DNA + 186μL of Tris EDTA buffer were mixed to create a 2ng/mL solution. 5μL of this solution was then added to the vial containing the BL21 cells. The vial was then left in ice to incubate. After incubation, the bacteria were subjected to a heat shock at 42˚C for 30 seconds. Shortly after the heat shock, 250μL of room temperature super optimal broth (SOB) medium was added to the vial and another 30 minutes of agitation occurred, but in a gyratory shaker instead. After the second agitation process, the mixture was added to a tube containing 10mL of Luria broth (LB) and 5μL of carbenicillin. This new solution was left to agitate over the whole night, so the bacteria with the desired protein expression can grow.

The next day, the solution was inoculated to 150mL of LB and antibiotic. The inoculated solution was then agitated for 3 hours on the gyratory shaker. Once this step was finished, 1x of Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the inoculated solution so protein expression can be induced. Growth by agitation was again induced for another 3 hours. After the final agitation, the solution was centrifuged for 10 mins at 3000xg. The precipitate formed from the centrifuge was then collected and frozen down in preparation for a lysate.

The precipitate from the previous day was pelleted into 8mL of guanidinium lysis buffer at pH 7.8. The solution was then mixed for 5-10 minutes. A freeze-thaw process was then performed with the solution. A mixture of dry ice and methanol was mixed in a beaker. The test tube containing the cells and the buffer were then placed into the dry-ice beaker, where the test tube had begun to completely freeze. Once the cell solution was frozen, the test tubes were put into a room temperature bath so the solution could be thawed out. This freeze-thaw process was repeated three times. After, the cells were subjected to sonication in which 15 second pulses

were conducted by the ultrasonic homogenizer machine for a total of one minute. The sonication procedure was repeated two-three times until the solution started to look clear. Shortly after the sonication, the cells were centrifuged for 15 minutes at 3000xg. Then, the newly formed lysate was transferred into another test tube to eliminate a possible precipitate from the centrifuge step. The lysate was frozen until the purification process could be conducted.

The Invitrogen ProBond Purification System was used to purify the SARS-Cov-2 N, S, RBD proteins. To begin the purification process, 2mL of Nickel (Ni) resin was pipetted into a purifying column. 6mL of water was then added, and the column was resuspended. The column was then centrifuged at 800xg for one minute and the supernatant was aspirated. Next, a 6mL denaturing binding buffer was added to the column, resuspended, centrifuged at the same settings previously used with the water, and the supernatant was then aspirated. This step was repeated with another 6mL of denaturing binding buffer. 8mL of the lysate that was made was placed into the purifying column and was agitated for 30 minutes, while making sure the contents were constantly resuspended inside. After agitation, the column was centrifuged at 800xg for one minute, and the supernatant was then aspirated. Next, the denaturing binding buffer step that was previously done was repeated twice, but with only 4mL of the buffer instead. After the same procedure of adding the buffer, resuspending, centrifuging, and aspirating was done again, but with denaturing wash buffer at pH 6.0 twice, followed by denaturing wash buffer at pH 5.3. Once the buffers had run through the column, 5mL of the elution buffer was added. The column was then clamped into a vertical position, the bottom cap was twisted off, and 1mL of the protein was collected into five different test tubes. Each test tube was then subjected to an OD280 reading to determine how much protein was in each 1mL sample. The three test tubes that had the highest OD280 readings were then combined into a singular test tube and the

solution was then dialyzed against a 10mM Tris pH 8 0.1% Triton X-100 solution overnight to remove any impurities. The next day, the dialyzed protein was then concentrated. This was done by first centrifuging the dialyzed protein at 3000xg for 10 minutes. Then the protein was placed in a concentrator tube and was centrifuged again for 10 minutes at 6000xg. The small amount of concentrated protein was then collected and frozen down into aliquots, ready for SDS page gel electrophoresis, and immunoblotting.

Protein Electrophoresis and Immunoblotting

Electrophoresis chamber was filled with a 1X running buffer up to the cathode. A 10% Tris-Glycine gel cassette was placed in the chamber after removing the comb and tape from it. More running buffer was added to the chamber, so the wells of the gel are just slightly filled. 8µL protein samples that were mixed and heated with another 8µL 2X loading dye were all placed in the wells accordingly. In the first lane of the gel, 8µL of molecular weight marker was placed so the size of the proteins can be determined when assessing. Running buffer was then added to the fill line and the electrophoresis had run for 90 minutes at 100V and 60mA.

After the electrophoresis had run, the protein needed to be transferred over to a PVDF membrane. Four Blotting sponges and two filters were soaked in 1X transfer buffer. The PVDF membrane was pre-soaked in methanol, then washed in water, and then finally soaked in 1X transfer buffer. To begin the transfer, a sandwich was made in the cathode module. Two sponges were first placed followed by a filter. Then the gel that was just made from electrophoresis was placed on top of the filter followed by the PVDF membrane. To close the sandwich, a filter paper was placed after and finally two sponges were added on top. The module was then closed by clamping the anode on top of the cathode. The module was then placed in the chamber and the

transfer was run for 90 minutes at 20V and 180mA. Once completed, the module was disassembled and the membrane, which was handled with tweezers, was placed in water for five minutes and then air dried and stored ready to be used for an immunoblot.

To begin immunoblotting, the PVDF membrane was first cut up into small strips so it would fit in individual lanes in the blotting tray. The strips were placed in methanol for a few seconds, and then washed in water. Then each membrane was placed in its individual lane and was submerged in 10mL of 5% blotto blocking buffer overnight. The next day, the blocking buffer was removed, and each lane was submerged again with their respective mixtures of blocking buffer and a 1:500 dilution of a primary antibody. The whole tray was then incubated for 2 hours while being agitated. After the 2 hours had finished, the primary antibody solution was removed and placed in a disposable beaker. Each lane was then washed with a wash buffer 3 times, where 3mL of the wash buffer was added and the whole tray would be rocked for two minutes. After the wash, each lane was submerged in a secondary antibody solution which contained 2mL of blocking buffer and a 1:2000 dilution of the secondary antibody. The tray was again incubated for 2 hours while being agitated. Once the 2 hours was over, the same wash procedure was repeated. Once the wash buffer was removed, each lane was submerged in a 2mL 1:1 Luminol:Peroxide substrate solution and incubation for 5 minutes was incurred. Then the membranes were placed inside the ChemiDoc imager and the immunoblotting protocol with high sensitivity was selected. Results were then shown on the screen.

Chapter III: RESULTS

Protein Purification

The method employed in this thesis was by purifying the lysate through histidine tagged affinity chromatography. The N, S, RBD proteins, that were subjected to purification, had six histidine tags attached to it on the Nitrogen terminus side. This was purposely done as when the lysate was subjected to be placed in the purification column, the histidine tags would be used as a target for the ligand in the resin used in this step. The resin consists of Ni2+ that has been immobilized with a chelating agent. The proteins that have the histidine tags will bind to the Ni2+ ligands, whereas the other proteins will not bind to the column or will bind weakly. Then, once all the protein has been bound onto the resin, elution occurs. The elution buffer contains imidazole, which competes with the histidine tags to bind with the charged nickel. The histidine tagged proteins will elute from the resin since the imidazole has a higher affinity to the charged metal as the imidazole ring mimics the side chain of histidine. Figure 3 shows a visual representation of how the purification works.

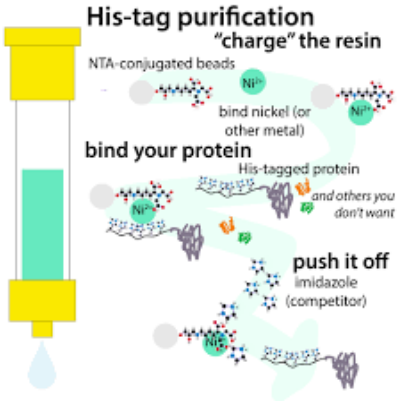

Figure 3. Visual representation of His-tag purification. The charged nickel (already charged in the resin) helps capture the his-tagged proteins only.

To assure the protein purification was successful, gel electrophoresis was conducted, and the gel was stained with Coomassie blue dye. Coomassie blue works by utilizing the ionic interactions between the positive amine groups of the protein and the sulfonic acid groups. All SARS-CoV-2 proteins that were purified were detected as bands on the gel with minimal nonspecific proteins. Figure 4 shows the N band appearing in between 40 kDA - 50 kDA since the protein size is 45 kDA. The S band appearing next around 70 kDA, which is the size of the S protein. The RBD band appears just under 25 kDA, which is the size of the RBD protein. This indicates protein purification was successful.

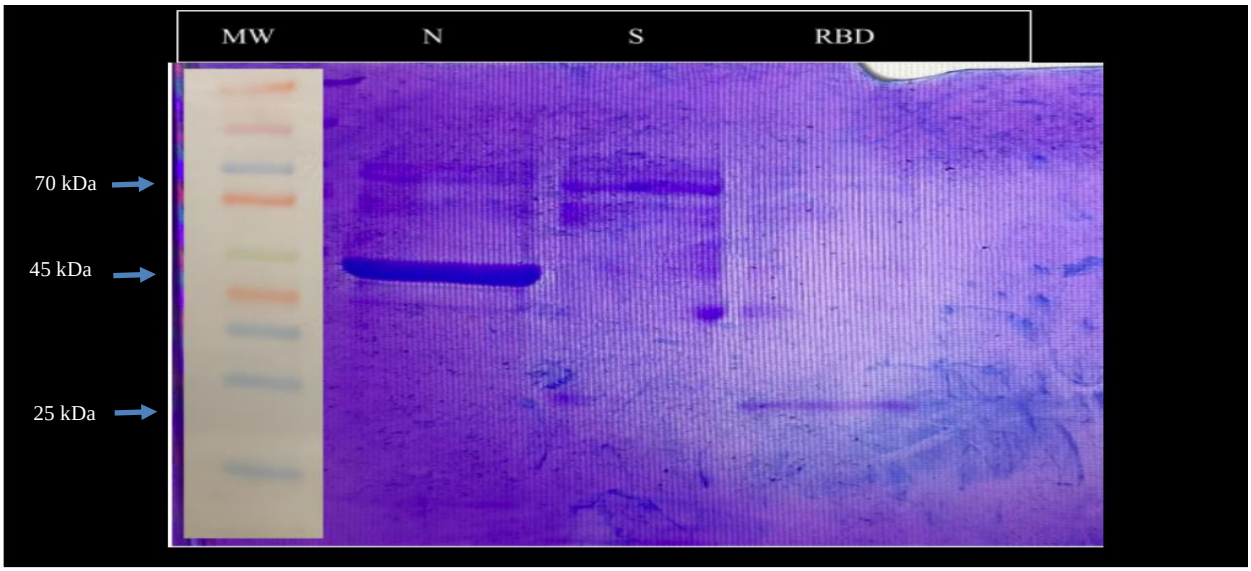

Figure 4. 10-20% tris-glycine gel analysis of protein purification. N, S, RBD bands appear on the gel. Molecular weight (MW) marker indicates size of the bands.

However, to make sure we truly got the proteins desired, rabbit sera was tested with all three proteins. The positive control of rabbit sera was derived by inoculation of rabbits with the SARS-CoV-2 N and S protein each by Sino Biological. This was done so the sera could then create the specific antibodies in accordance with the respected proteins. The negative control rabbit sera were just regular rabbit serum that had no association with SARS-CoV-2 proteins.

Therefore, the primary antibody used was an anti-rabbit SARS-CoV-2 nucleocapsid for the N protein, and an anti-rabbit SARS-CoV-2 Spike for the S and RBD protein for the positive controls, and normal rabbit serum for negative controls. The secondary antibody used was a goat anti-rabbit IgG peroxidase for both controls. Figure 5A shows the S protein band in the first lane compared to the RBD protein band in the second lane. The size of the first band is around 70 kDa, whereas the second band is around 25 kDa. Next to that blot is the negative control for both of those proteins. The negative control does have some sort of spillage, however there are no distinct bands compared to the positive control. From this result, the antibodies that were present had attached to the purified S and RBD on the membrane. Therefore, the proteins were able to bind on to the FaB region of the primary antibody, and the secondary antibody used was able to bind onto the Fc region of the primary antibody (Janeway, Travers & Walport, 2001).

Figure 5B indicates the presence of the N protein on the left membrane, whereas the right membrane indicates no binding of antibodies. The dark band seen has a size around 40kDa indicating the presence of the N protein. The immunoblot shows the respective molecular weight bands of each protein. This is a confirmation that the observed bands were indeed purified SARS-CoV-2 proteins since the rabbit antibodies were made specifically to target each individual protein. With all three bands present at the correct molecular weights, we can correctly identify and conclude that the SARS-CoV-2 N, S, and RBD proteins have all been purified and transferred onto the PVDF membrane.

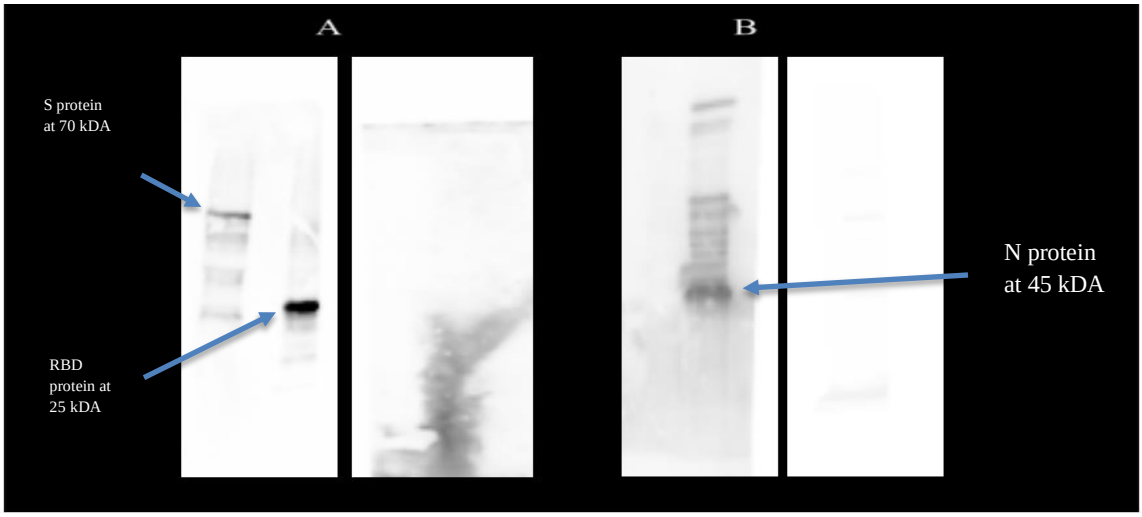

Figure 5. Immunoblot analysis of rabbit sera. Primary antibody rabbit anti-rabbit SARS-CoV 2 S (Sino Biol. 1/500), anti-rabbit SARS-CoV-2 N (Sino Biol. 1/500), or normal rabbit serum. Secondary antibody goat anti-rabbit peroxidase (1/2000).

Detection of antibodies to the SARS-CoV-2 proteins in human sera

Finally, human sera were tested with a positive and negative control for all three proteins as well. All human sera were obtained from RayBiologicals. Figure 6 shows five lanes. Each lane had sera from different individual patients. Therefore, sera from five different individuals were used. This immunoblot was conducted to see if human antibodies would bind to the presence of the N protein. The first three lanes were incubated with human patient sera who all were previously infected with COVID-19. Therefore, these patients should produce antibodies to the N protein due to the previous exposure to the SARS-CoV-2 virus, and thus act as a positive control for the experiment. The last two lanes were induced with human sera that did not have any contact with SAR-CoV-2. Therefore, the last two lanes will act as a negative control as in theory, no antibodies should be produced from the exposure to the SARS-CoV-2 proteins. The infected human patient sera for the first three lanes, and the non-infected human sera for the last

two lanes all acted as a primary antibody for this immunoblot. Then all five lanes were incubated by the same secondary antibody (goat anti-human IgG horse radish peroxidase), which was obtained from Invitrogen. The secondary antibody is conjugated to horse radish peroxidase (HRP) as it reacts very well. HRP reduces hydrogen peroxide to water by the usage of Amplex red as an electron donor. This reaction leads to a fluorescent light as a byproduct, which can be picked up from the Chemidoc imaging machine to observe the bands on the membrane. The first three lanes of Figure 6 all show clear distinct bands around the 45 kDa mark. This indicates that human antibodies were able to bind to the N protein of SARS-CoV-2. Small bands above and below the positive lanes are seen, however this may be non-specific binding that could have been caused from a too long of an exposure to the secondary antibody during the experiment. The negative control lanes show no signs of binding indicating the expected outcome.

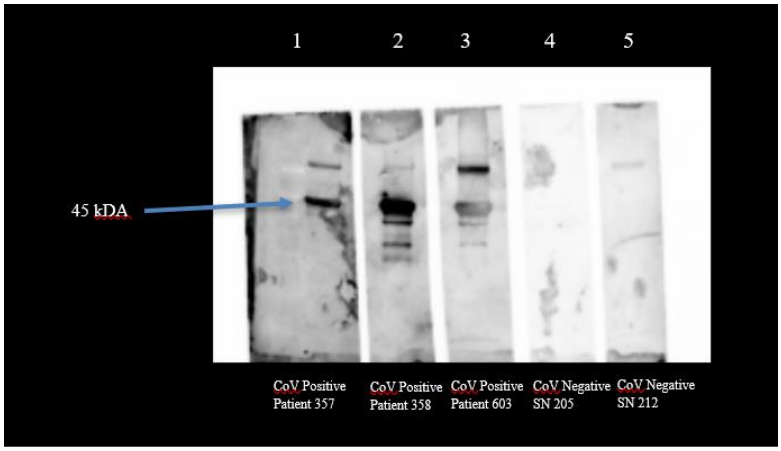

Figure 6. Immunoblot analysis of human sera for N. Primary antibody - serum from a SARS-CoV-2 infected human (1/500, Ray Biologicals)
Secondary antibody- goat anti-human IgG horse radish peroxidase (1/2000).

Figure 7 is an immunoblot to the S protein. The immunoblot has four lanes. The first three being positive controls, and the last one being a negative control lane. The first three lanes were all incubated with human patient sera that were all infected with COVID-19, and the last

lane was reacted with human patient sera that had no previous exposure to SARS-CoV-2. All five lanes were also induced with goat anti-human IgG horse radish peroxidase after initial exposure to the primary antibody. The positive control lanes all show a molecular band around 75 kDa. The second positive control lane shows the same band; however, it is very faint but indication of it being a positive control is still concluded. The positive and negative control lanes all have some non-specific binding bands but none around the 75 kDa area. As a result, human antibodies were able to bind to the S protein of SARS-CoV-2. The positive control lanes used in this immunoblot had the same sera from the positive control lanes in Figure 7. Therefore, each human patient's sera interact differently as seen by the immunoblot.

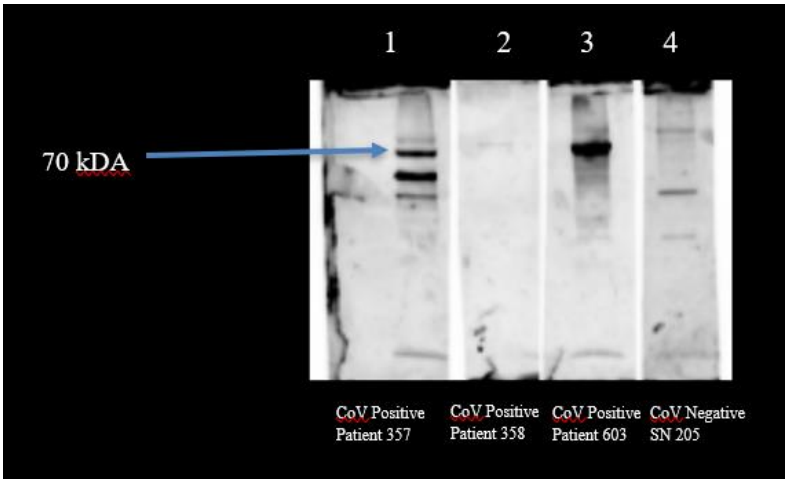

Figure 7. Immunoblot analysis of human sera for S. Primary antibody - serum from a SARS-CoV-2 infected human (1/500, Ray Biologicals)
Secondary antibody goat anti-human- HRP peroxidase (1/2000).

Figure 8 shows very interesting results. This immunoblot was conducted to the exposure of the RBD protein. The first three lanes in Figure 8 are positive control lanes to the RBD protein, and the last two lanes are negative controls. Once again, the first three lanes were incubated with human patient sera who all were previously infected with COVID-19 and the last two lanes were incubated with human sera that did not have any contact with SAR-CoV-2. All 5

lanes were reacted with goat anti-human IgG horse radish peroxidase. Although the expected outcome for the negative controls appeared on the immunoblot, the positive controls did not show any protein bands. This is very unusual as the rabbit sera was able to bind to the RBD, but the human sera were unsuccessful. For all three positive controls to not bind to the RBD protein may indicate to us that using the RBD protein for an immunoblot may not be efficient in comparison to the N and S protein.

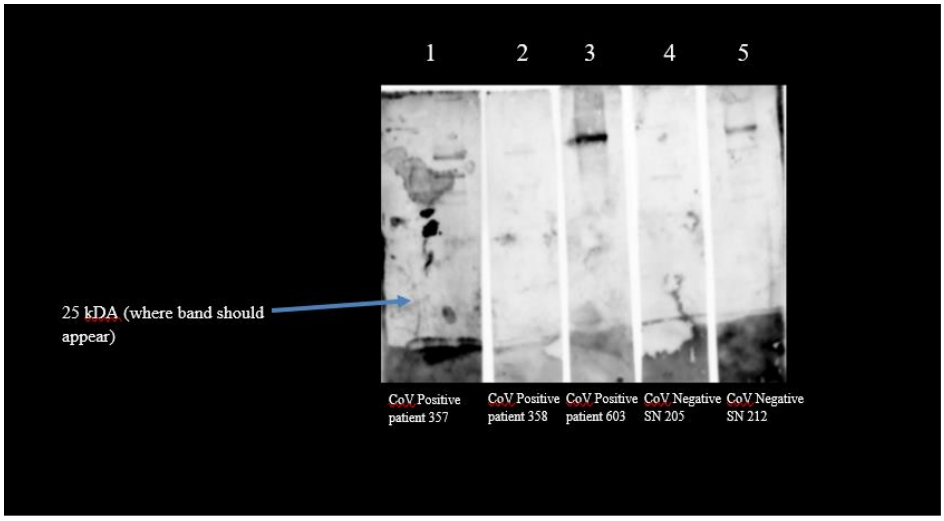

Figure 8. Immunoblot analysis of human sera for RBD protein. Primary antibody - serum from a SARS-CoV-2 infected human (1/500, Ray Biologicals)
Secondary antibody goat anti-human - HRP peroxidase (1/2000).

CONCLUSION AND FUTURE EFFORTS

Overall, an immunoblot was developed to detect human antibodies against proteins from the SARS-CoV-2 virus. Even though the human antibodies were not able to be detected from the presence of the RBD protein, the protocol developed was successful in identifying human antibodies from the N and S proteins. Furthermore, the protocol for purification and transfer of the N, S, and RBD proteins was successful. Data collected from the rabbit sera immunoblot, and the tris-glycine gel show that the viral proteins were purified and that antibodies were able to successfully bind to the proteins. To strengthen this claim, observations from the rabbit sera immunoblot and the tris-glycine gel show that the bands were at the expected molecular weight size, indicating the presence of the protein. As a result, the first goal of this thesis was met, as a protocol was created to purify and transfer the viral proteins for usage of immunoblots.

The second goal of evaluating human antibody response to the SARS-CoV-2 proteins in this thesis has been partially met. Out of the three proteins in study, only two were successfully able to be recognized by human antibodies – the N and the S protein. The RBD protein was not shown to have any signs of interaction with human antibodies. An explanation for this maybe that human antibodies are not able to recognize the RBD protein unlike the rabbit antibodies. However, the SDS page gel used is a denaturing gel. In theory, the structure of the RBD protein may be altered in such a way that human antibodies are unable to recognize this altered shape. Therefore, the antibodies are not able to bind. Another theory may be that the virus may employ a mechanism that hides the RBD protein from the host's defenses. The RBD protein is integral for surface attachment to the host. If this structure is compromised, then the virus itself will fail its goal to

replicate and survive within a host. As a result, evasive measures may be employed by the virus to avoid jeopardizing the structural integrity of the RBD protein.

Overall, the results indicate that individuals that may have been exposed to the virus in the past can be identified through this procedure. The ELISA assay can indicate false positives from exposure to non-specific binding of antibodies to the viral antigens in the wells, causing the color change associated with the assay. This can be detrimental as patients will then believe they have the active immunity against the SARS-CoV-2 virus, but they have no specific means of targeting the virus. With the immunoblot, the specific molecular weight where the bands are looked for will show a more conclusive result since we are now identifying the binding interactions at a specific weight rather than a color change. The importance of this is that recognizing these individuals will prevent further transmission of the virus, as they can begin to take preventative measures accordingly such as a vaccine. Therefore, using the immunoblot as an antibody test will aid in the diagnosis of patients and the test can be served as a preventative measure for patients.

Future experiments with additional human sera may be conducted to further investigate the binding of antibodies to purified N and S proteins, and maybe even the RBD protein.

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