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THE DETECTION OF HUMAN ANTIBODIES AGAINST DIFFERING SARS-COV-2 VIRUS PROTEINS BY IMMUNOBLOT TESTING

by
Virginia Kate Gammon

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 2022

Approved by

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ABSTRACT

VIRGINIA KATE GAMMON: THE DETECTION OF HUMAN ANTIBODIES AGAINST DIFFERING SARS-COV-2 VIRUS PROTEINS BY IMMUNOBLOT TESTING
(Under the direction of Dr. Wayne L. Gray)

The emergence of the coronavirus disease (COVID-19) has instigated one of the most influential pandemics, causing unique and detrimental effects throughout various parts of the world. By developing a stronger understanding of the viral genome and structural components, researchers are driven towards medical advancements and the implementation of improved testing methods. This research aims to provide a reliable COVID-19 antibody test by immunoblot detection, which can deliver more accurate results than the currently used enzyme linked immunosorbent assay (ELISA). Within this protocol, differing SARS-CoV-2 viral proteins were detected through western blotting analysis and were evaluated based upon differing strengths in antibody detection. The SARS-CoV-2 spike (S) glycoprotein and nucleocapsid (N) protein were expressed within an *E. Coli* histidine tag plasmid and subjected to column purification. The purified proteins were fractionated by SDS-PAGE gel electrophoresis and transferred to a PVDF membrane by western blotting. The separated membranes were exposed to primary antibodies, gathered from human and rabbit sera, and secondary antibodies, gathered from goat anti-human and goat anti-rabbit IgG Horse Radish Peroxidase Conjugate. Detection by chemiluminescent western blotting confirmed the presence of antibodies to the S and N proteins within positive SARS-CoV-2 patient sera, and the absence of

antibodies to the S and N proteins within SARS-CoV-2 seronegative sera. The produced imaging displays the strength of antibody detection against both proteins, promoting the use of the viral nucleocapsid protein as a valuable target for antibody detection. This study conclusively demonstrates the reliability of antibody detection by immunoblot testing and promotes the implementation of western blotting analysis in future COVID-19 research efforts.

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LIST OF ABBREVIATIONS

1°	Primary
2°	Secondary
Ab	Antibody
ACE2	Angiotensin Converting Enzyme 2
BB	Blocking Buffer
℃	Degrees Celsius
COVID-19	Coronavirus Disease 2019
dH2O	Distilled Water
E	Envelope
E. Coli	Escherichia Coli
ELISA	Enzyme Linked Immunosorbent Assay
FDA	Food and Drug Administration
IPTG	Isopropyl β-d-1-thiogalactopyranoside
M	Membrane
M	Molar
mA	Milliampere
mg	Milligram
µg	Microgram
µl	Microliter
mL	Milliliter
mM	Millimolar
MtOH	Methanol

MW	Molecular Weight
N	Nucleocapsid
NAAT	Nucleic Acid Amplification Testing
NaOH	Sodium Hydroxide
ng	Nanogram
O.D.	Optical Density
PVDF	Polyvinylidene Difluoride
RBD	Receptor-Binding Domain
RNP	Ribonucleoprotein
rpm	Revolutions Per Minute
RT-PCR	Reverse-Transcription Polymerase Chain Reaction
S	Spike
SARS	Severe Acute Respiratory Syndrome
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
V	Volt
WHO	World Health Organization

INTRODUCTION

The novel coronavirus, identified as COVID-19, has led to one of the world's largest and most influential pandemics throughout our current period. The SARS-CoV-2 virus, causing COVID-19 disease, has produced over 454 million cases and a current total of 6 million deaths, worldwide. The emergence of this pandemic has severely impacted various regions of the world and has produced a dramatic influence upon global healthcare systems. As this new disease has become the central focus of medical research, the entire world has worked towards the development and the improvement of testing and prevention methods, in an attempt to resolve this healthcare crisis.

The current method of antibody testing is through enzyme linked immunosorbent assay (ELISA), with a central focus upon the detection of the spike (S) glycoprotein. Overtime, the accuracy of this testing method has been brought into greater question, due to a rising statistical measure of false results. False positive results have granted potentially infected patients a sense of viral immunity, leading to increased public exposure and viral transmission. An improvement within antibody testing methods can eradicate the probability of false results and provide accurate confirmation of the detection of patient antibody.

This study was conducted to improve current antibody research and detection methods, through the development of an efficient COVID-19 immunoblot assay. A western blot analysis can be used to detect the presence of antibodies to the SARS-CoV-2 spike (S) glycoprotein and the SARS-CoV-2 nucleocapsid (N) protein. It is hypothesized

that the development of a COVID-19 immunoblot assay will provide more reliable results, relative to ELISA, and that the detection of the SARS-CoV-2 nucleocapsid (N) protein will produce a new target for future antibody testing.

CHAPTER 1: BACKGROUND ON COVID-19

Emergence of COVID-19

While the vast majority of the world lived free of fear surrounding the spread of new diseases, global perceptions on emerging pandemics were suddenly altered within the final months of 2019. Throughout the city of Wuhan in China's Hubei Province, various cases were reported by individuals experiencing pneumonia-like symptoms. While there have been multiple theories formulated regarding the origin of this outbreak, initial beliefs argued that potential viral hosts were sold within the Huanan seafood wholesale market (You, 2020). Investigators identified both snakes and bats as potential hosts, with evidence more strongly supporting the claim of origination within the bat species. Following exposure within the wet market, fifty initial cases reported symptoms of severe acute respiratory syndrome (SARS), which led to the onset of further varying symptoms. With a population of 11.1 million citizens, the crowded conditions within the city of Wuhan allowed for rapid transmission (Muralidar, 2020). Individuals with more severe symptoms underwent hospitalization, where testing was completed by the Chinese Center for Disease Control and Prevention. The control center used throat swab testing to classify this as a coronavirus disease. With further investigation, the World Health Organization (WHO) identified the novel virus as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and named the disease caused by the virus coronavirus disease 2019 (COVID-19) (You, 2020). As cases began to expand towards distant villages within the Hubei Province, WHO declared this a public health emergency

worldwide. The disease crept towards the nation's boundaries, bringing the possibility of global transmission to a daunting reality.

Only three weeks after the initial Wuhan outbreak, South Korea, Japan, and the United States each confirmed their first cases (Brahma, 2020). With a rapid rate of progression throughout expanding nations, the entire world gradually fell victim to the grueling impacts of this disease. While each nation implemented differing techniques of intervention and regulation, WHO was able to monitor the impact of prevention programs upon the wave of expansion throughout different regions of the world. Efforts for prevention struggled to combat expansion, as the entire world would eventually face deadly impacts. With outreach to 225 countries and territories throughout the world, cases have skyrocketed to a cumulative estimate of 454 million confirmed cases and over 6 million deaths (Worldometer, 2022). As COVID-19 continues to leave damaging impacts upon our current society and the world's healthcare system, medical advancements and scientific research continue to fight for a healthier future.

Viral Transmission

COVID-19 is transmitted between individuals through the spread of infected respiratory droplets, which can be found on surfaces or in the air. Close contact with infected individuals allows for inhalation of small liquid particles, spreading from the infected person's breathe, sneeze, cough, etc. (CDC, 2022). The infected aerosols are in greatest concentration within three to six feet of the infectious source, which allows for short-range airborne transmission. Additionally, people can be infected by touching

contaminated surfaces prior to touching their mouth, eyes, or nose. The virus can then be introduced to the throat's mucous membranes.

Initial research strongly supported the idea that transmission originated in the animal products within the Wuhan meat markets. This theory gained further support with evidence of animal transmission from other viruses within the family, *Coronaviridae*. MERS-CoV and SARS-CoV caused two preceding global outbreaks, due to transmission from differing species of rodents and bats (Bergmann, 2020). Many of the original COVID-19 patients experienced only fifteen to fifty seconds of exposure within the market and public places, prior to testing positive (Muralidar, 2020). Although research found a correlation between a multitude of original cases and patient attendance to the meat market, some of the earliest patients claim to have neither attended nor eaten products from this market. This finding brought to light further considerations of differing modes of transmission. A discovery of human to human transmission debunked previous theories of animal to human transmission. With an incubation period as short as two days, the disease allowed for rapid and easy transmission between individuals. Mask regulations, proper social distancing, and the implementation of preventative techniques have helped to combat further transmission.

Disease Progression, Mutations, & Variants

With rising expansion across the world, COVID-19 triggered viral growth and propagated through replication. As with many other RNA viruses, researchers were able to predict gradual changes to the coronavirus genome (Bollinger, 2022). However, they did not know when these changes would occur, how the virus would evolve overtime, nor

how these viral changes could impact pathogenicity. Within genetic replication, there is a growing possibility for copying errors. An increasing number of copying errors within the COVID-19 genome introduced the possibility for changes to viral structure, making antigens unrecognizable to antibodies (Bollinger, 2022). These mutations will be introduced as variants to the original strain. What has been identified as the strongest genetic modification is structural alterations to the virus' spike protein, possibly allowing it to bind more strongly to our cells.

With geographical isolation, viruses are more strongly inclined to develop such modifications, introducing new strains in unexpected areas of the world. Although multiple variants have formed, the quickest spreading variants have been identified as the delta and omicron variants. In December 2020, concerns for the identification of India's delta variant were brought to the attention of WHO and CDC (Brahma, 2020). These organizations defined this as a variant for concern, due to its increasing rate of transmission. The delta variant's rapid transmission ultimately led to greater rates of hospitalization and death, and would later be identified as the most contagious form thus far (Bollinger, 2022). In November 2021, South Africa's omicron variant was identified as an additional variant of concern. The WHO reported a shorter incubation period, which contributes to the increased transmissibility within this variant. Although vaccines have been reported to decrease the rate of infection for these variants, the creation of new variants in the future could introduce new problems within the healthcare system. Additionally, it is difficult to predict how these structural modifications can impact the accuracy of future COVID-19 testing. The possibility for future complications shines light upon the importance of research to examine and monitor these ongoing changes.

Symptoms

Within the initial stages of the pandemic, social perceptions regarding the severity of symptoms stemmed from mass hysteria and a fear of the unknown. With time, research has shown that COVID-19 patients range from being asymptomatic to experiencing severe symptoms. Signs of SARS-CoV-2 infection can appear within two to fourteen days after exposure (CDC, 2022). Although the incubation period can range over a span of days, presymptomatic transmission can allow individuals to spread COVID-19 prior to exhibiting initial symptoms. The most common symptoms are fever, cough, loss of taste or smell, headache, sore throat, myalgia, diarrhea, chills, etc. (Muralidar, 2020). Additional severe symptoms can include pressure in the chest, acute heart injury, difficulty breathing, pneumonia, etc.

Although the majority of patients will experience only mild symptoms, older individuals are at a higher risk of experiencing severe symptoms and serious illness. Additionally, a variety of unhealthy life habits and prior medical conditions can worsen potential severity. Such medical conditions can include cancer, diabetes, heart disease, dementia, down syndrome, liver disease, pregnancy, etc. (Bergmann, 2020). Unhealthy lifestyle habits, such as smoking and being overweight, have contributed to the growing risk of hospitalization due to COVID-19.

With the emergence of differing variants, the CDC has noted changes in symptom severity and the type of symptoms presented. The incubation periods for both the delta and omicron variants have grown shorter, allowing for a more rapid spread. Due to quicker transmission, the rate of hospitalization consequently increased. Researchers found that symptoms of the delta variant appeared to be very similar to the original

COVID-19 infection (Bollinger, 2022). Additionally, the CDC has noted that fewer individuals infected with the omicron variant are likely to have severe illness, such as pneumonia.

Diagnosis & Testing

Following recent exposure or the onset of initial signs of infection, individuals are highly encouraged to request a COVID-19 viral test. The two types of tests that target current infection include molecular tests and antigen tests (FDA, 2022). Molecular tests, which use patient saliva or a nasal swab, are able to provide more reliable results. These tests commonly use Nucleic Acid Amplification Testing (NAAT) or Reverse-Transcription Polymerase Chain Reaction (RT-PCR) assays. To detect the SARS-CoV-2 infection, the RT-PCR molecular test will identify the specific RNA viral pattern, amplify the genomic sequence of interest, and generate an output approximation of the amount of amplified genomic sequences (FDA, 2022). Using this measurement, the test will clarify whether the viral sequence is found within the patient's sample.

Antigen tests, which can also be referred to as rapid tests, are used to detect certain proteins within the structure of the virus. The rapid tests commonly use the nucleocapsid protein as a target for detection of the viral antigen. Using the sample from a nasal swab, rapid tests can provide results within fifteen to thirty minutes. Although testing results are provided at a much quicker rate, these tests have been found to be less reliable, due to risk of false-negative results. The antigen tests are used to detect the proteins within the viral structure. These tests are considered to be effective between one to five days after the onset of symptoms. The amount of antigen found within the sample

will decrease the longer the individual has had symptoms. If the results come back negative, yet the patient still possesses symptoms, it is recommended to complete PCR testing for more accurate results (CDC, 2022). In addition to viral tests that target current infection, antibody tests are used to detect antibodies from previous infection or from vaccination.

Treatment & Quarantine

Upon receiving positive results, it is very crucial for the patient to self-isolate as quickly as possible. Within the emergence of COVID-19, individuals were initially advised to self-isolate for fourteen days following exposure or the onset of symptoms. Isolation has been effective in reducing the spread of infection and can decrease the rate of hospitalization. Depending upon the viral variant, the time of quarantine can vary (Bollinger, 2022). For example, individuals infected with the omicron variant have been recommended to isolate for only five days, following their first positive test.

It is still important for individuals to isolate after exposure to an infected individual, even if they are asymptomatic or were provided negative test results (CDC, 2022). These individuals should isolate for five days after exposure, in order to prevent potential transmission.

Current studies indicate that there are limitations to the use of antiviral therapeutic agents for the treatment of COVID-19. The current care for COVID-19 infection is to provide supportive treatment by managing only the symptoms. The only prescription medicine offered are two drugs, Remdesivir and Paxlovid, which are capable of only alleviating severe symptoms (Mariano, 2020). For individuals who are experiencing such

symptoms, oxygenation, fluid management, and ventilation can additionally be administered (Muralidar, 2020). Patients who have undergone hospitalization should commonly have their heart rate and oxygen levels monitored. Infected individuals with less severe symptoms are recommended to self-care by monitoring their symptoms closely, staying hydrated, getting rest, and maintaining isolation.

Vaccines

In addition to the distribution of antiviral medications, the invention of the COVID-19 vaccine has represented a breakthrough within our healthcare system and has saved many lives around the world. Within one year, the Pfizer vaccine gained emergency use authorization from the Food and Drug Administration (FDA) and was first released on December 11, 2020. The German immunotherapy company, BioNTech, and Pfizer started working together on the development of the vaccine in March (Silver, 2022). One of the key components within the production of the Pfizer vaccine was the insertion of cationic lipid nanoparticles, which provide protection to the mRNA. The mRNA delivers COVID-19 viral genetic material, which can be used to train the cells within our bodies to produce the spike protein. Our immune system will use this protein, or antigen, to produce antibodies that fight COVID-19 infection. Through rapid production, Pfizer was able to reach a goal of creating the vaccine by the end of 2020 (Silver, 2022). Using data from the initial clinical trials, the results proved the company's invention to be ~95% effective at preventing infection from the COVID-19 virus. Two shots are injected in the muscle of the upper arm, twenty-one days apart (CDC, 2022).

The two Pfizer facilities located their main production plants in Puurs, Belgium, and Kalamazoo, Michigan, allowing for greater distribution throughout Europe and the United States (Silver, 2022). Each state was granted the ability to decide who the first recipients of the Pfizer vaccine would be. Most states sought to provide the first vaccinations to healthcare providers, elderly populations, those at higher risks of severe infection, immunocompromised, and patients throughout hospitals or healthcare centers. Very shortly after the release of the Pfizer vaccine, other companies, such as Moderna and Johnson and Johnson, gained FDA approval to release their original vaccines. Overtime, the rapid distribution by multiple vaccine companies granted the world easier access to receive vaccination. By increasing the number of vaccinated individuals within the world, the risk of getting and spreading the virus that causes COVID-19 has significantly declined. The development of the COVID-19 vaccination has saved lives worldwide and has formed an optimistic outlook upon the future of COVID-19.

With the introduction of new variants, researchers have found a partial decrease in the effectiveness of the original vaccines. However, the invention of the COVID-19 booster shot helps to provide additional protection against infection and prevent severe symptoms (Bollinger, 2022). Although the COVID-19 vaccine will not completely inhibit an individual from becoming infected, the CDC still highly recommends individuals aged five years or older to receive a vaccination, in order to decrease risk of infection, to decrease the severity of potential symptoms, and to help protect others against COVID-19.

CHAPTER II: BACKGROUND ON ANTIBODY TESTING

COVID-19 Structure & Proteins

SARS-CoV-2 exists within the *Coronaviridae* family, under the order of *Nidovirales*. Coronaviruses can be further classified by genera, with COVID-19 identified as a beta-coronavirus (Muralidar, 2020). Through research, scientists have gained a far better understanding of the structural components within the virus, leading to strong advancements in biotechnology. With a proper understanding of the physical characteristics and protein identifications, biotechnologists can work through a means of prevention, such as the invention of vaccines that target specific proteins. Coronaviruses have positive, single-stranded RNA genomes, which are surrounded by a capsid and an envelope (Mariano, 2020). 29,891 nucleotides, which encode for 9,860 amino acids, are found within the single strand (Muralidar, 2020). The positive, single-stranded genetic material contains a 3'-poly-A tail and a 5'-cap. Within the 5'-end of the virus, a frameshift between Orf1a and Orf1b leads to the formation of two polypeptides which produce sixteen non-structural proteins. On the 3'-end of the strand, four structural proteins and nine accessory proteins are encoded, which impact the virulence. The structural proteins include the nucleocapsid (N) protein, the spike (S) glycoprotein, the membrane (M) protein, and the envelope (E) protein (Mariano, 2020). The viral envelope is formed by the S, M, and E proteins, while the N protein can be found within the internal packing of the envelope. The nucleocapsid protein is able to bind to the RNA genome, and is essential to the internal packing process.

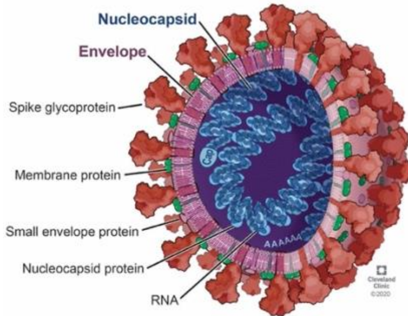

Figure 1: Structure of Coronaviruses – This figure demonstrates the location of the differing structural proteins, relative to the nucleocapsid, RNA, and viral envelope. The single positive-sense RNA is 26 – 32 kb (similar to the host mRNA). (https://www.ccjm.org/content/87/6/321)

The name of the coronavirus originated from its crown shape appearance, which is formed by multiple spike (S) glycoprotein projections upon the virus' surface. The spike protein is an essential component within the virus' ability to invade the host cell. This S protein consists of two subunits, S1 and S2, which are formed through cleavage. The S1 subunit resides within the genomic N-terminal, which contains the receptor-binding domain (RBD). The RBD is essential for binding to angiotensin converting enzyme 2 (ACE2), the host cell receptor. Following the binding of ACE2 to the RBD, the S2 subunit is cleaved by the host serine protease, causing dissociation of S1 and S2 (Mariano, 2020). A conformational change within S2 allows for fusion between the host membrane and viral structure. The S protein displays a fundamental role in SARS-CoV-2 viral entry, which has been a basis of research within the field of biotechnology. Many vaccines and testing strategies have been invented that use the spike protein as a direct target within prevention efforts. Although the spike glycoprotein does play a pivotal role in SARS-CoV-2 viral infection, other structural proteins should be strongly considered for further evaluation within prevention efforts and in research advancements.

The nucleocapsid (N) protein is a structural protein found within the internal surface of the viral envelope, where it is in direct contact with the viral genetic material. Here, it plays a critical role in the packaging of viral RNA within ribonucleoprotein (RNP) complexes (Mariano, 2020). Although the RNP complexes are not in contact with the other structural proteins, they are able to bind to the N protein and form a preferred orientation with two juxtaposed curved walls. In addition to its role in viral packaging, the N protein plays an important role in viral genome replication. Within the replication transcription complex, Nsp3 will recruit the N protein. Through joint N-terminal activity between the N protein and Nsp3, the structural protein is used to direct the viral genome to the replication complex. The roles performed by the multifunctioning N protein display the level of activity occurring within the internal compartment of the COVID-19 structure.

The envelope (E) protein is positioned within one trans-membrane domain, where it is able to interact with itself and other structural proteins (Mariano, 2020). The E protein functions to form ion channels and is additionally important within the assembly of virions. The membrane (M) protein can be found within the viral membrane. Positioned through three transmembrane helices, its primary function leads to the gathering of new virions within the host cells. The M protein is additionally responsible for recruiting other structural proteins and represents a scaffolding platform (Mariano, 2020). Although these structural proteins interact and work in a collective manner, they each provide an independent and pivotal role in the formation and/or function of the SARS-CoV-2 virus.

Current Antibody Detection Methods

SARS-CoV-2 antibodies can be detected in individuals who have recently been infected with the virus that causes COVID-19 and within individuals who have received vaccination. Following exposure to the SARS-CoV-2 virus, the white blood cells within our immune systems begin to produce protective proteins, known as antibodies. These proteins are used to build immunity to the specific virus, and are able to fight against future infection. Antibodies will recognize, bind to, and destroy the specific viral antigen. For individuals who have recently been infected, it can take their immune system one to three weeks to produce and secrete SARS-CoV-2 antibodies within the bloodstream (CDC, 2021). An alternative way to activate antibody production to the SARS-CoV-2 virus is through vaccination. By exposing the body to the specific virus, vaccination will lead to the production of antibodies and provide immunity against the virus.

The COVID-19 antibody test, or serology test, is a recently used method of detecting antibodies against the SARS-CoV-2 virus. Although these do not test for active infection, they were initially produced to measure one's level of viral immunity. Enzyme linked immunosorbent assays (ELISA) are provided by most local healthcare facilities or can be completed within a laboratory setting. This assay attempts to detect antibodies within patient serum, specifically to the SARS-CoV-2 spike glycoprotein.

Upon initial distribution of the COVID-19 antibody test, many individuals relied upon the results as a means of measuring one's level of immunity. By providing positive antibody results, this test afforded individuals a sense of protection against future infection and led to the misperception that one cannot contribute to the further spread of this virus. Through statistical analysis, researchers have detected a number of false results

and have gradually lost a sense of reliability upon the accuracy of ELISA. In May of 2021, the FDA confirmed that the public should not rely upon the results of the SARS-CoV-2 antibody test as a way to measure one's level of immunity (FDA, 2021). This administration discourages individuals from using the provided antibody assays as a means of evaluating their level of protection, as false positive results have led to false beliefs and the further spread of the virus. Through public interaction, individuals that mistakenly depend upon these misconceptions can fall target to potential infection or can perilously expose others to the virus.

Through recent studies, a more reliable method of antibody detection has been identified and can be used to eliminate false positives within clinical results. The implementation of an immunoblot assay, also referred to as western blot analysis, during completion of the antibody test will provide a more reliable means of measurement compared to current methods. By assessing the efficacy of western blotting, a more effective method of antibody detection can be conducted and analyzed, in an effort to advance future COVID-19 research and to prevent the further spread of the SARS-CoV-2 virus.

Reasoning Behind Focus on Nucleocapsid Protein Within Detection

Within this study, the method of western blotting analysis is demonstrated to show a higher rate of efficiency in antibody detection. The currently used ELISA tests are found to provide inaccurate results, and are hypothetically less reliable than the immunoblot antibody tests. Throughout recent antibody testing, researchers have relied upon the detection of the spike protein for accurate antibody results. However, my study

argues that other proteins can be used for antibody detection. The nucleocapsid (N) protein possesses a unique location within the viral structure, where it comes into direct contact with the RNA viral genome. Unlike the other structural proteins, the N protein holds an interior position and resembles the core of the viral structure. By examining the close connection that this protein shares with the virus' genetic material, it can be hypothesized that this structural protein is of greatest potential within antibody testing and should be analyzed for detection of a more interior component of the virus. This study compared the level of detection between the spike (S) glycoprotein and the nucleocapsid (N) protein, conducted through the more reliable antibody testing method of western blot analysis.

Purpose of Research

The purpose of this experiment is to provide a more efficient means of antibody detection, through the development of an immunoblot assay. This technique can be used to eliminate false positive results, which are commonly reported within ELISA antibody testing. Following analysis of the immunoblot assay's efficiency, researchers could potentially use this method within future research efforts and biomedical practices. The implementation of this test can confirm the presence of antibodies to both the SARS-CoV-2 spike (S) glycoprotein and the SARS-CoV-2 nucleocapsid (N) protein. These results will provide a measurement of the strengths of spike protein detection and nucleocapsid protein detection through antibody testing. Although the spike protein is the current staple for antibody detection, it is hypothesized that the nucleocapsid protein is a valuable target for testing and should be considered in future research.

CHAPTER III: METHODS

Protein Expression & Purification

Prior to completion of antibody testing, the specific protein must be expressed and purified properly. This protocol will detail the steps of expression and purification of both the N protein and the S protein, which were specifically designed for the purpose of this experiment.

Transforming E. Coli with Nucleocapsid or Spike DNA

Within the initial phase of this experiment, *E.Coli* BL21 star cells were transformed with pET 100 TOPO SARS-CoV-2 Nucleocapsid (or Spike) DNA. To begin, the vial of chemically competent Invitrogen *E. Coli* BL21 star cells was thawed on ice. Using a pipette tip, PET 100 SARS-2NC Clone 7 DNA was diluted to 2 ng/ml. The plasmid, which is a circular piece of DNA, was inserted into the bacterial cell and utilized within the synthesis of protein expression. 5 µL of the diluted DNA was added to the BL21 vial, which then incubated in ice for 30 minutes. The vial underwent heat shock at 42℃ for 30 seconds, and was then returned to ice. This heat shock caused the breakdown of the cell wall, which allowed the plasmid to enter the cells. 250 µL of room temperature sterile SOC medium was added to the vial, which then incubated with agitation for 30 minutes (200 rpm, 37℃). The mixture was added to 10 mL of LB media. 5 µL of carbenicillin (100 mg/mL, 2000x concentration) was added to a final concentration of 50 µg/mL. The modified bacteria then grew overnight at 37℃.

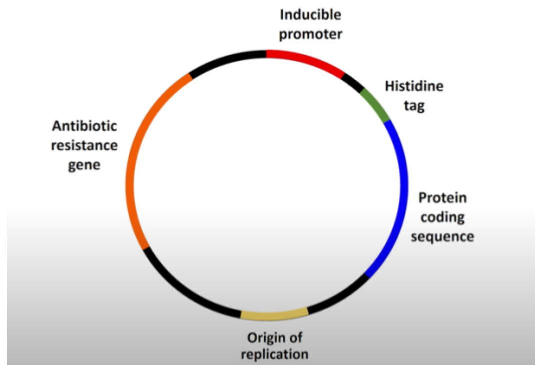

Figure 2: Histidine Tag Protein Expression Plasmid – This figure shows the plasmid that was inserted within the cells. The antibiotic resistance gene was utilized to select for the specific bacteria that contains the plasmid. The inducible promoter provided the ability to choose when the protein of interest would be expressed. (https://www.youtube.com/watch?v=YJr_Xtji5NE&t=52s)

Expressing N or S Protein

Following bacterial growth overnight, 150 mL of LB was inoculated with carbenicillin (75 µL of 2000x stock – final 50 µg/mL) in the overnight culture. The carbenicillin antibiotic was used for bacterial cell wall degradation, through inhibition of cell wall synthesis. This mixture was then agitated for 2 – 3 hours at 37℃ to an $O.D._{600}$ of 0.5 – 0.8. After agitation, protein expression was induced by adding 1.5 mL of IPTG (100, M, 100x) to a final concentration of 1mM. The inducer (IPTG) removed the repressor from the lac operon to induce protein expression. Following induction, this solution incubated at 37℃ for 4 hours with 200 rpm shaking. In order to separate the cells from the media, this solution was centrifuged at 3000 x g for 10 minutes. This high speed spinning was used for separation of the bacterial cells, based upon density differences. Following separation, the supernate was removed and the cell bacterial pellets were frozen overnight at -80℃.

Preparing Cell Lysate

The next phase of protein expression began with equilibration of Guanidium Buffer to a pH of 7.8. Once equilibrated, 8 mL of the buffer was added to the cell pellets. The cell pellets were rocked for 5 – 10 minutes. Using an acetone-dry ice bath, the solution was freeze thawed three times. This opened the cells, allowing the protein of interest to break away. To complete further cell lysis, we used a sonicator, which applied ultrasonic sound waves to the solution. This energy agitated and disrupted the cells, causing the cell membranes to break apart (Martínez, 2020). The sonication protocol was completed four times, with 15 second bursts at an amplitude of 60. Following sonication, the solution was centrifuged at 3000 x g for 15 minutes, to separate the proteins from the cells. Finally, the supernate was transferred to new tubes and frozen overnight at -80℃.

Column Purification

To begin the final stage of protein purification, 2mL of nickel resin was pipetted into the column, allowing gravity to make it settle at the bottom for 5 – 10 minutes. By loading the supernatant onto the column, the protein of interest bound to the nickel resin. The nickel resin contained the histidine tag that was encoded during protein formation. 6 mL of sterile water was then added. This mixture was resuspended and the resin was allowed to settle. The proteins that were not bound to the nickel resin were able to flow freely through the column. Once settled, this was centrifuged at 800 x g for 1 minute and the supernatant was aspirated. 6 mL of denaturing binding buffer (pH 7.8) was added to the solution. This mixture was resuspended and the resin was allowed to settle. This was centrifuged again at 800 x g for 1 minute and the supernatant was aspirated. 8 mL of the protein lysate was then added to the column and the resin was resuspended. This was

bound for 15 – 30 minutes with gentle agitation. Again, this solution was centrifuged at 800 x g for 1 minute and the supernatant was aspirated. The column was then washed with 4 mL of the denaturing binding buffer, agitated for 2 minutes, resuspended, and the resin was allowed to settle. This was centrifuged at 800 x g for 1 minute and the supernatant was then aspirated. The column was washed with another sample of denaturing wash buffer, which had a pH of 6.0. The following steps of agitation, resuspension, settlement, centrifugation, and aspiration were repeated. The column was washed with an additional sample of denaturing wash buffer, which had a pH of 5.3. The listed steps were repeated. Through multiple cycles of washing the column, this technique ensured that the unwanted proteins, which were not bound to the nickel resin, were removed from the column. A sample of 5mL of elution buffer (pH 4.0) was then added to the solution. The elution buffer, which contained imidazole, removed the histidine tag from the nickel, allowing the protein of interest to be collected from the column. The protein was eluted into 5 test tubes, each containing 1 mL. Using Nanospot spec, I completed an $O.D._{280}$ reading of each sample and measured the adsorption of my protein of interest.

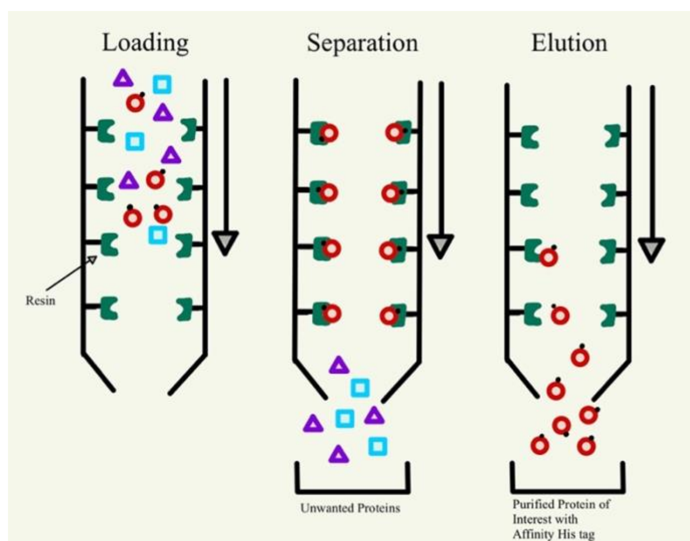

Figure 3: Protocol For Column Purification – This figure shows the three steps of column purification. The first column was loaded with a mixture of proteins. The protein of interest was identified with an affinity histidine tag bound to it. By adding the denaturing binding buffer to the column, it separated the protein of interest from the unwanted proteins. A strong affinity between the His-tag and the nickel resin allowed the protein of interest to bind within the column. In the final step, the elution buffer was added to the column, allowing the His-tag protein to unbind from the nickel resin. The purified protein was then collected.
Figure constructed by author.

Table 1: Optical Density Results

Tube 1	0.797 mg/mL
Tube 2	1.127 mg/mL
Tube 3	0.632 mg/mL
Tube 4	0.266 mg/mL
Tube 5	0.192 mg/mL

Considering that the first three tubes contained the most purified forms of the protein, the final two tubes were discarded. The first three test tubes were dialyzed overnight against 500 mL of 10 mM Tris pH 8 0.1% Triton X – 100, using a Thermo Scientific Slide-A-lyzer cassette (10 K cut-off), in order to remove the urea. A buoy was used to float the dialyzer. The next day, the sample was removed from the dialyzer, using

a 10 mL syringe. A Pierce Protein Concentrator further concentrated the dialyzed materials. For further protein isolation, I centrifuged 3 mL of the sample at 3000 x g for 5 minutes and transferred it to the concentrator. This concentrated sample was then centrifuged at 6000 x g for 10 minutes, and reduced to a volume of 0.5 mL. Finally, 50 - 100 µL of the aliquots was frozen at -80℃. Following protein purification, the resin was cleaned by washing it with 0.5 M NaOH for 30 minutes. The resin was then equilibrated in denaturing binding buffer and stored at 4℃ for later use.

Protein Electrophoresis & Western Blotting (Chemiluminescent)

This protocol includes the steps that are required to run a protein gel electrophoresis, which is utilized for isolation of proteins within a lysate. This details the steps to blot the lysate onto the polyvinylidene difluoride (PVDF) membranes, which can then be used for detection of the protein of interest (N or S protein). We can image these proteins via chemiluminescent substrates on the BioRad ChemiDoc digital imager.

Protein Electrophoresis

To begin, the electrophoresis chamber was filled with 1X running buffer. The comb and tape were removed from the 10% Tris-Glycine gel cassette, which was inserted into the clamp and locked into a raised position. Within an ice bath, the protein of interest (N or S protein), a 2X sample buffer, and a molecular weight (MW) marker were chilled, until later use. Once moderately thawed, a 30 µL sample of the protein was pipetted into a new test tube. To denature the protein of interest, the 30 µL test tube was placed in a 95℃ water bath for 3 minutes. Once denatured, the 30 µL sample of the protein of interest was combined with 30 µL of 2X sample buffer, at a 1:1 ratio. The MW marker

and the protein of interest (with 2X sample buffer) were loaded within their designated wells (Figure 4). Once the cassette was properly assembled, protein electrophoresis ran for 75 minutes at a constant 100 V (60 mA). Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins based upon their molecular masses.

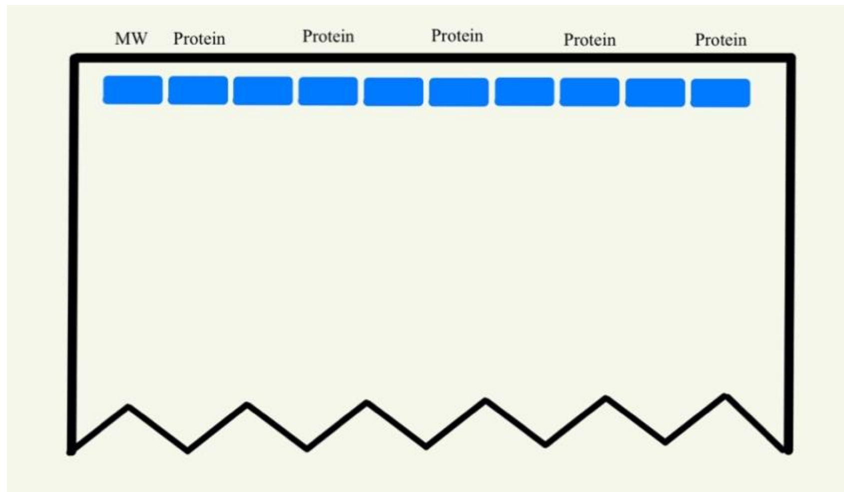

Figure 4: Sequence For Loading Wells Prior to Performing SDS-PAGE – Within the first well, 8 μL of Spectra Multicolor Broad Range Protein Ladder was inserted. Within the second, fourth, sixth, eighth, and tenth wells, 12 μL of the protein of interest:2X sample buffer was added to the gel.
Figure constructed by author.

Western Blotting

Once protein electrophoresis was completed, the assemblage of the western blotting chamber began. Using tweezers, a PVDF membrane was placed in a MtOH Tupperware tray for 30 seconds. The PVDF membrane was briefly washed with dH20, and then submerged in a 1X transfer buffer Tupperware tray for 3 – 5 minutes. Simultaneously, the gel cassette was removed from the electrophoresis chamber, and opened using a gel knife. Once opened, the top and bottom segments of the gel were trimmed. Within the module, two soaked sponges, a filter paper, and the gel were placed

onto the cathode. Using tweezers, the PVDF membrane was carefully aligned on top of the gel. A soaked filter paper and two additional soaked sponges were placed on top of the membrane. The bubbles were rolled out of the sandwich, and the anode was clamped on top of the cathode. Once tightly closed, the blotting module was placed into a chamber, filled with 1X transfer buffer. The blotting transfer ran for 90 minutes at a constant 20V (180 mA). Once completed, the PVDF membrane was washed with dH20, and cut into individual testing strips. These strips were transferred into a Ziploc bag and refrigerated at 4℃, until later use.

Chemiluminescent Protein Detection

To wash the separate membrane lanes, they were placed in MtOH and then dH2O. Within Perfect Western trays, the separate membranes were blocked overnight in 10 mL of 5% blotto blocking buffer (BB), or non-fat milk solution. Once blocked, the BB was removed from each tray. Each lane was filled with 1:500 primary antibody in 5% BB (4 μL specific 1° Ab : 2 mL of 5% BB). These primary antibodies were collected from positive and negative human sera, and from anti-nucleocapsid or anti-spike rabbit sera. The soaked lanes were rocked for 4 hours (40 rpm, 25℃). Using wash buffer, the membranes were washed multiple times. Each lane was filled with 1:2000 secondary antibody in 5% blotto blocking buffer (1 μL specific 2° Ab : 2mL of 5% BB). These secondary antibodies were dependent upon the 1° Ab species (Goat anti-human IgG Horse Radish Peroxidase Conjugate) (Goat anti-rabbit IgG Horse Radish Peroxidase Conjugate). The soaked lanes were rocked for 2 hours (40 rpm, 25℃). These membranes were thoroughly washed again with wash buffer. After removing the wash buffer, the membranes were taken downstairs, where they were soaked in a 1:1 ratio of Luminol :

Peroxide Substrate (2 mL per lane) for 90 seconds. The reaction between the substrate and the enzyme (Horse Radish Peroxidase) promoted detection by emitting light through the release of photons (Figure 5). Once incubated, the membranes were removed from the substrate and partially dried. They were then placed on the cellophane sheets of the BioRad ChemiDoc imager, which was set to "Western Blotting protocol with high sensitivity detection." This system exposed the membranes to light, while capturing digital images that displayed specific MW bands. By comparing the size of the detected protein to the molecular weight ladder, this test was able confirm whether the specific antibody bound to the protein of interest. Protein detection through chemiluminescence has provided sufficient and accurate results, contributing to a more efficient method of antibody testing.

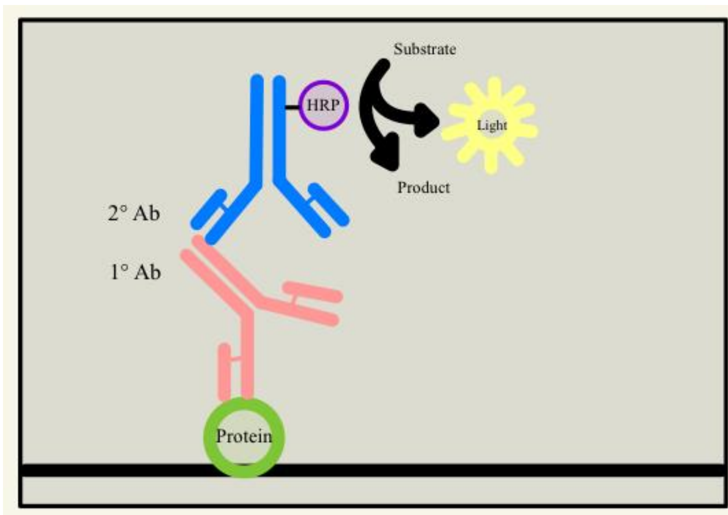

Figure 5: Chemiluminescent Western Blotting – This figure displays the mechanism that promotes detection within Chemiluminescence. If the antibody is present, it will bind to the protein of interest and will be detected in imaging. However, when the antibody is not present within the patient sera, the protein will not be able to bind to anything and will therefore not appear in imaging. The illustrated reaction between the substrate and the enzyme (HRP) is responsible for the emission of light and the production of a signal. Figure constructed by author.

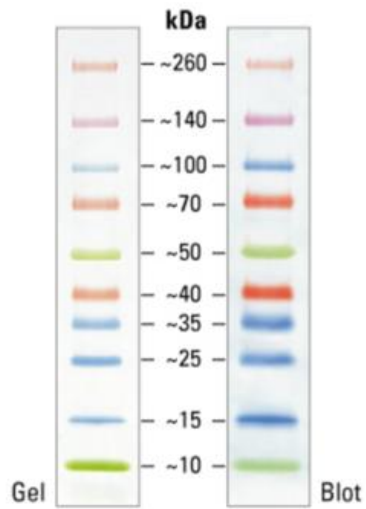

Figure 6: Image of a 10% Tris-glycine Gel (SDS-PAGE) – This figure provides the band profile of the Spectra Multicolor Broad Range Protein Ladder, which is used to identify and separate proteins detected in chemiluminescence by molecular weight differences.
(https://www.thermofisher.com/order/catalog/product/26634)

CHAPTER IV: COVID ANTIBODY RESPONSE & DETECTION

Controls & Discussion

Within the initial phase of this experiment, control tests were run to ensure the proper efficiency of the methods and materials used within this protocol. The rabbit anti-nucleocapsid and rabbit anti-spike sera were purchased from Sano Corporation. The preliminary detection showed that the rabbit anti-spike sera was able to bind properly to the purified SARS S1 protein and that the rabbit anti-nucleocapsid sera was able to bind properly to the purified SARS N protein. Following immunoblot imaging, the MW ladder was used to identify the differences in the signals provided by both proteins (Figure 6).

A positive control confirmed that the SARS S1 protein was purified in *E. coli* as detected by rabbit anti-SARS S antibody (Figure 7). A signal was identified at the 70 kD mark, demonstrating that this immunoblot technique provided sufficient results for antibody detection. A positive control provided valuable results, which confirmed the SARS N protein was purified in *E. coli* as detected by rabbit anti-SARS N antibody (Figure 8). These results prove that the nucleocapsid protein can be used as an efficient target for antibody detection. The N protein was detected with a clear image and a strong signal at 45 kD. The strong results provided by both positive controls support the implementation of this protocol on human sera.

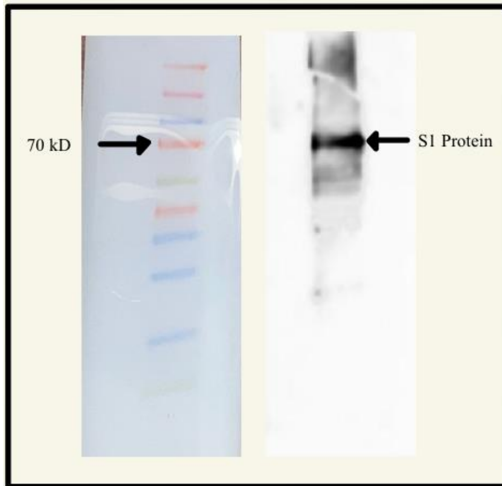

Figure 7: Western Blot Analysis Confirms SARS-CoV-2 S1 Protein Expression in *E. Coli* as Detected by Rabbit Anti-SARS S Antibody - This figure shows a positive control, using Rabbit Anti-Spike Serum.

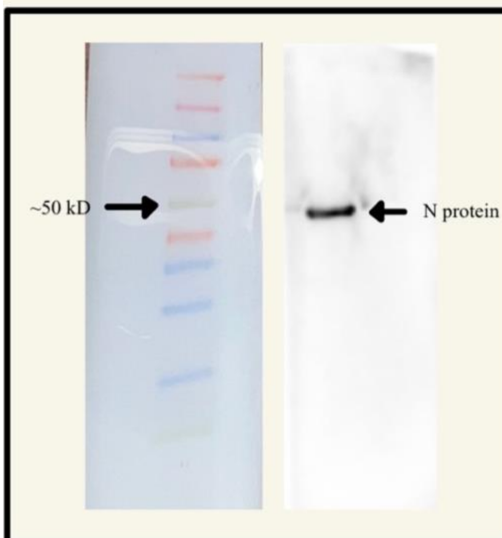

Figure 8: Western Blot Analysis Confirms SARS-CoV-2 N Protein Expression in *E. Coli* as Detected by Rabbit Anti-SARS N Antibody - This figure shows a positive control, using Rabbit Anti-Nucleocapsid Serum.

Human Sera Results & Discussion

Within the second phase of this experiment, the protocol was completed using human sera from COVID positive and COVID negative (negative control) patients. The results provide a measure of efficiency for western blotting analysis in proper human antibody testing and can be used to compare the strengths of detection between the

SARS-CoV-2 S1 protein and the SARS-CoV-2 N protein. Ray Biotech provided human sera, gathered from seronegative patients and from COVID-19 patients, confirmed by RT-PCR, antigen testing, or ELISA.

The immunoblot proved that SARS-CoV-2 infected humans produced antibodies to the SARS-CoV-2 S1 protein (Figure 9). Human sera received from negative patients (Figures 9A & 9B) was used as a negative control within this protocol. Antibodies to the SARS-CoV-2 S1 antigen were not detected in the sera of COVID negative patients. These results demonstrated that the spike protein was not detected within the absence of COVID antibodies. Human sera received from positive patients (Figures 9C, 9D, & 9E) confirmed the presence of antibodies through detection of the SARS-CoV-2 S1 protein during imaging. These positive results confirm that the spike protein will only be detected in the presence of COVID antibodies. The protocol was repeated for detection of human antibodies to the SARS-CoV-2 S1 protein with other positive and negative human-sera samples.

The immunoblot proved that SARS-CoV-2 infected humans produced antibodies to the SARS-CoV-2 N protein (Figure 10). Human sera samples received from negative patients (Figures 10A & 10B) were used as negative controls within this protocol. Antibodies to the SARS-CoV-2 N antigen were not detected in the sera of COVID negative patients. These results demonstrated that the nucleocapsid protein will not be detected within the absence of COVID antibodies. Human sera samples received from positive patients (Figures 10C, 10D, & 10E) confirmed the presence of antibodies through the detection of the SARS-CoV-2 N protein during imaging. These positive results confirm that the nucleocapsid protein will only be detected in the presence of

COVID antibodies. The protocol was repeated for detection of human antibodies to the SARS-CoV-2 N protein with other positive and negative human-sera samples.

Each test provided accurate results through proper detection of antibody presence or absence. Fifteen out of the fifteen COVID positive sera samples used for nucleocapsid protein detection provided positive results, while thirteen out of the thirteen COVID positive sera samples used for spike protein detection provided positive results (Table 2). Six out of the six COVID negative sera samples used for nucleocapsid protein detection provided negative results, while four out of the four COVID negative sera samples used for spike protein detection provided negative results (Table 3). The results represent a measure of efficiency for the proper detection of antibody presence or absence.

These results strongly support the implementation of immunoblot assays for accurate antibody detection. Both positive and negative patient sera results possessed a high level of accuracy, promoting this method of detection as a means to eradicate false results within the future. Additionally, this experiment supports a redirection of focus towards the nucleocapsid protein within future antibody research. Although the spike protein did provide accurate signal detection, the nucleocapsid protein also produced clear and defined MW bands. These precise signals support the hypothesis that the nucleocapsid protein should be utilized for future antibody testing. The N protein's internal position and stronger association with the viral genetic material provides a deeper and more accurate means of detection for antibody presence.

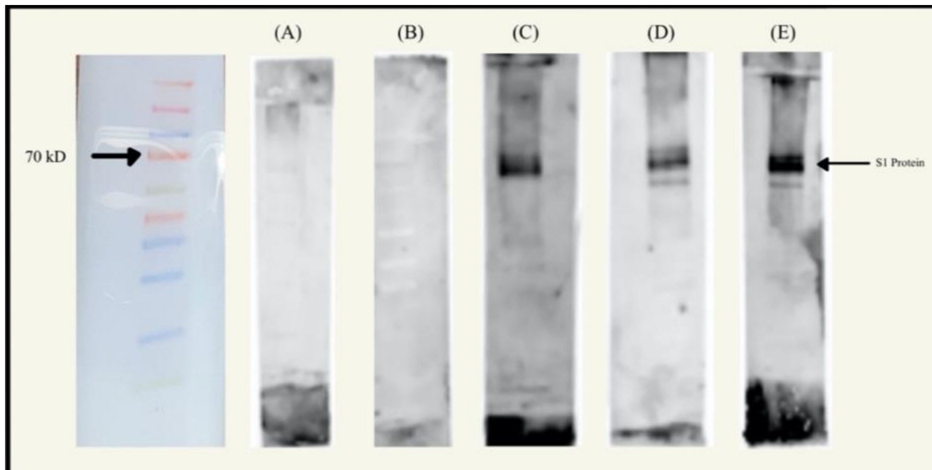

Figure 9: Western Blot Analysis to Prove That SARS-CoV-2 Infected Human Produces Antibody to the SARS-CoV-2 S1 Protein – This figure shows the results for Immunoblot Antibody Detection from Human-Sera. Patient (9A) (CoV Neg Patient 204) and Patient (9B) (CoV Neg Patient 205) were both used as negative controls within this experiment. Patient (9C) (CoV Pos Patient 310), Patient (9D) (CoV Pos Patient 313), and Patient (9E) (CoV Pos Patient 602) were used as positive controls within this experiment. These results confirm 70 kD SARS-CoV-2 S1 Protein Expression in *E. Coli* as detected by Human Anti-SARS S Antibody.

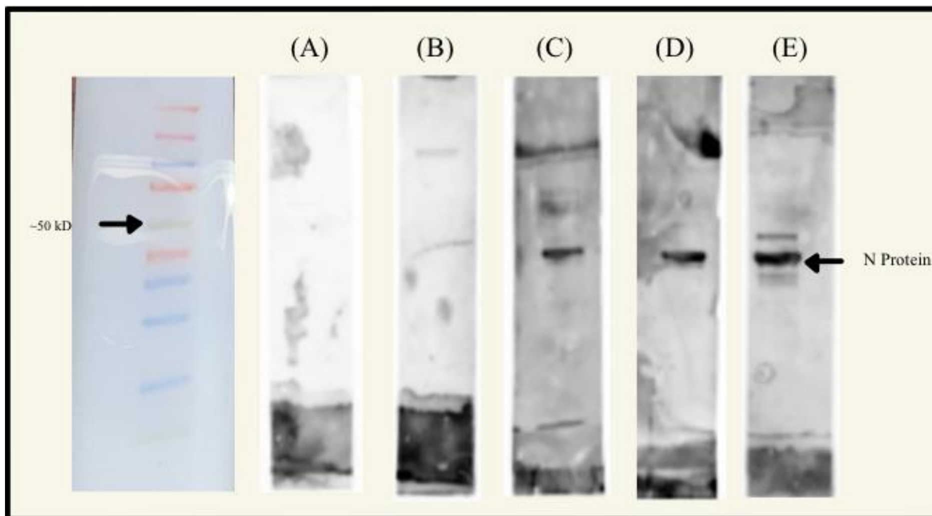

Figure 10: Western Blot Analysis to Prove That SARS-CoV-2 Infected Human Produces Antibody to the SARS-CoV-2 N Protein – This figure shows the results for Immunoblot Antibody Detection from Human-Sera. Patient (10A) (CoV Neg Patient 205) and Patient (10B) (CoV Neg Patient 212) were both used as negative controls within this experiment. Patient (10C) (CoV Pos Patient 346), Patient (10D) (CoV Pos Patient 333), and Patient (10E) (CoV Pos Patient 303) were used as positive controls within this experiment. Relative to Figure 9, the N protein was found below the S1 protein. These results confirm 45 kD SARS-CoV-2 N Protein Expression in *E. Coli* as detected by Human Anti-SARS N Antibody.

Table 2: Results from Positive Human Sera

Testing dates within the second and third columns are provided based upon the respective patient and protein of interest.

(+) symbol within column 1 signifies a positive COVID patient serum sample, purchased from Ray Biotech and confirmed by RT-PCR, antigen testing, or ELISA.

(+) symbol within columns 2 and 3 signifies a positive immunoblot detection.

Patient	SARS-CoV N Ab	SARS-CoV S Ab
302+	+ 9-2-21	
303+	+ 9-2-21	+ 10-14-21
308+	+ 1-28-22	
310+	+ 11-16-21	+ 10-12-21 + 10-14-21
313+	+ 8-24-21	+ 10-28-21
314+	+ 11-16-21	+ 10-12-21
333+	+ 4-9-21	+ 10-21-21
346+	+ 4-9-21	+ 10-21-21
347+	+ 4-9-21	+ 10-21-21 + 11-10-21
348+	+ 1-28-22	
351+	+ 9-2-21	
357+	+ 3-1-21	+ 3-4-21
358+	+ 3-1-21	+ 11-10-21
602+	+ 9-2-21	+ 11-10-21
603+	+ 3-1-21	+ 3-4-21

Table 3: Results from Negative Human Sera (Negative Control)
Testing dates within the second and third columns are provided based upon the respective patient and protein of interest.
(-) symbol within column 1 signifies a seronegative patient serum sample, purchased from Ray Biotech and confirmed by RT-PCR, antigen testing, or ELISA.
(-) symbol within columns 2 and 3 signifies a negative immunoblot detection.

Patient	SARS-CoV N Ab	SARS-CoV S Ab
204-	- 4-9-21	- 10-28-21
205-	- 3-1-21	- 10-28-21
208-	- 4-9-21	- 10-28-21
211-	- 1-28-22	
212-	- 3-1-21	- 10-28-21
213-	- 1-28-22	

CHAPTER V: CONCLUSION & FUTURE EFFORTS

Through the development of an effective COVID-19 immunoblot assay, this study has provided a reliable means of antibody detection, which can be used to eradicate false results commonly produced by current testing methods. This protocol used rabbit sera and human sera for detection of antibodies against the SARS-CoV-2 spike (S) glycoprotein and the SARS-CoV-2 nucleocapsid (N) protein. Within our negative control samples, the immunoblot assay did not provide spike protein nor nucleocapsid protein signal detections. Human sera received from COVID positive patients confirmed the presence of antibodies during imaging of both spike and nucleocapsid proteins. This confirms COVID-19 immunoblot assay detection only in the presence of COVID-19 antibodies. These results support implementation of this antibody test in future research efforts. Although ELISA has been the commonly implemented method of detection, western blotting analysis is more accurate. Advantages of ELISA testing are the simplicity and the speed of the procedure. However, the lower level of accuracy of ELISA testing is a significant disadvantage. An additional benefit presented by western blotting analysis is the ability to detect specific proteins, an advantage not obtainable by ELISA. The study's results also exhibit the value of the nucleocapsid (N) protein as a target for future antibody detection. Although the spike protein is of central use within current antibody detection, the nucleocapsid protein's internal position and close relation to the viral genetic material makes it a promising focus for detection. By comparing N protein and S protein detection, chemiluminescent imaging proved the nucleocapsid

protein is of equal value within future research efforts. Through the detection of both the N protein and the S protein within antibody testing, patients will be provided more dependable results. The primary goal of this experiment was to work towards the improvement of current COVID-19 antibody research methods, in order to eliminate false results and to provide patients with a more reliable sense of immunity. Through the development of a more efficient antibody testing assay, future research efforts could prevent the further spread of disease and one day achieve liberation from the COVID-19 pandemic.

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