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Development of Recombinant Varicella-Zoster Virus Vaccines Expressing SARS-CoV-2 Antigens

by
Cody Blake Wilson

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

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ABSTRACT

The COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus (SARS-CoV-2) continues to cause infections and deaths around the globe. Since its emergence, scientists have worked diligently to discover and produce vaccines to battle the virus. While they successfully created and distributed vaccines, the vaccines are not without flaw. These vaccines do not grant long term immunity, require multiple booster shots, and have not prevented the emergence of viral variants. In this study, the SARS-CoV-2 receptor binding domain (RBD), which is a key part of the spike protein (S), and nucleocapsid (N) genetic sequences were inserted into the live varicella zoster virus (VZV) vaccines creating a live recombinant (rVZV) expressing SARS-CoV-2 antigens (rVZV-SARS-Cov-2).

The overall goal of this project was to confirm rVZV-SARS-CoV-2 vaccines express SARS RBD and N antigens. Specific aims of the experiment were to confirm that the SARS RBD and N genetic sequences are located in open reading frame 13 of the rVZV-SARS-CoV-2 vaccines and to confirm expression of SARS RBD and N antigens in rVZV-SARS-CoV-2 infected cells by immunoblot and immunofluorescence assay.

The rVZV-SARS-Cov-2 vaccines were shown to contain the genetic sequence for both the RBD and N antigens by PCR and to express the SARS-CoV-2 RBD and N antigens in infected cells by immunoblot and immunofluorescence assay. Based on these results, the rVZV-SARS-CoV-2 RBD and rVZV-SARS-CoV-2 N vaccines may proceed to animal testing with the hope of reaching clinical trial.

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LIST OF ABREVIATIONS

dH_2O……Distilled Water

E……..……Envelope

EUA……...……Emergency Use Authorization

FDA……..……Food and Drug Administration

J&J……...Johnson and Johnson

kD ……Kilodalton

M……...……Membrane

MeWo……...……Human Melanoma

N……...……Nucleocapsid

NBCS……...……Newborn Calf Serum

PCR……Polymerase Chain Reaction

RBD…… Receptor Binding Domain

RT-PCR…….……Reverse Transcriptase Polymerase Chain Reaction

rVZV-N……Recombinant Varicella Zoster Virus Vaccine with Nucleocapsid Genetic Sequence

rVZV-RBD……Recombinant Varicella Zoster Virus Vaccine with Receptor Binding Domain Genetic Sequence

rVZV-SARS-CoV-2…...……. Recombinant Varicella Zoster Virus Expressing Severe Acute Respiratory Syndrome Coronavirus Antigens

rVZV-SARS-N…….……Recombinant Varicella Zoster Virus Severe Acute Respiratory Syndrome with Nucleocapsid Genetic Sequence

rVZV-SARS-RBD…….……Recombinant Varicella Zoster Virus Severe Acute Respiratory Syndrome with Receptor Binding Domain Genetic Sequence

rVZV……...…….……Recombinant Varicella Zoster Virus

S……Spike

SARS-CoV-2……..……Severe Acute Respiratory Syndrome Coronavirus

SDS-PAGE……...…Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis

VZV……Varicella Zoster Virus

wtVZV…….……Wild-Type Varicella Zoster Virus

INTRODUCTION

The COVID-19 pandemic will go down in history as one of the most impactful disease outbreaks in the history of mankind. Due to its rapid transmission and the potential for severe symptoms, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has led to the infection of just over 683,000,000 people and the deaths of 6,829,000 people worldwide as of March 2023 ("Coronavirus Cases"). In order to try to slow its spread, many countries shut down travel, schools, and business that were labeled as non-essential. Despite these precautions, hospitals were quickly overrun by the onslaught of infected individuals. Medical grade masks and ventilators were in short supply forcing hospitals to allocate them to sicker patients. While hospitals worked tirelessly to treat patients, scientists around the world worked around the clock to create a vaccine to slow down the virus. In under a year, a mRNA vaccine was developed and successfully reduced the transmission, morbidity, and mortality of the disease; however, the vaccines were far from perfect.

Current vaccines have assisted humanity greatly in dealing with the COVID-19 pandemic, but they have not eradicated the disease. These mRNA vaccines do not grant long term immunity for the virus which is why constant booster shots are required for the immune system to maintain a defense. Also, the vaccines focus only on one protein, the spike (S) protein, which has led to the evolution of the many variants of SARS-CoV-2. The many steps required to maintain immunity and the newness of the vaccine led people to be skeptical of it.

This research aims to provide a vaccine that effectively combats SARS-CoV-2 with long-term immunity and ends the hesitancy for vaccination. By using the varicella zoster virus (VZV) vaccine, the chickenpox vaccine, as a safe and effective vaccine vector, live recombinant varicella zoster virus severe acute respiratory syndrome coronavirus (rVZV-SARS-CoV-2)

vaccines were constructed to either express the SARS-CoV-2 receptor binding domain (RBD) or nucleocapsid (N). By using a live vaccine, the immune system is able to sustain long term immunity like it does when an individual is naturally infected by a virus. With long term immunity, there will be no need for multiple booster shots. By expressing both the RBD and N antigens of COVID-19, the virus will not evolve as quickly to evade the immune system which will lower the chance of variants. The use of VZV as a vector for the RBD and N genetic sequence will allow people to feel more comfortable with vaccination. If the rVZV-SARS-CoV-2 vaccines can successfully grant strong, long-term immunity and reduce hesitancy in vaccination, it can potentially end the threat of the COVID-19 disease.

Chapter 1: What is COVID-19?

Coronavirus

Over the last several years, people across the world have called the pandemic that brought the world to a standstill the coronavirus. However, the name coronavirus actually is the name for an entire family of viruses that have distinct spike proteins on their surface. These different coronaviruses can range in severity from symptoms of the common cold to much more severe symptoms. For example, Middle East Respiratory Syndrome branches from the coronavirus family with a fatality rate of 34.4% (Pustake, 2022). While there are many coronaviruses, SARS-CoV-2 caused the pandemic that is known as COVID-19.

SARS-CoV-2

While it is unknown, the leading theory for the origination of SARS-CoV-2 is the transmission of bats to humans which occurred in Wuhan, China, in December of 2019 (Yee, 2021). The virus itself is composed of four structurally significant proteins that have various functions. The outermost layer of COVID-19 is the S protein which attaches to receptors on the surface of human cells to allow viral penetration. Specifically, the RBD of this protein binds to receptors on the surface of human cells. The following layers are the viral membrane (M) and viral envelope (E) that serve to protect the virus's internal RNA genome. The final structural portion of COVID-19 is the N protein. This protein serves the role of providing a protective coat directly around the RNA genome (Figure 1).

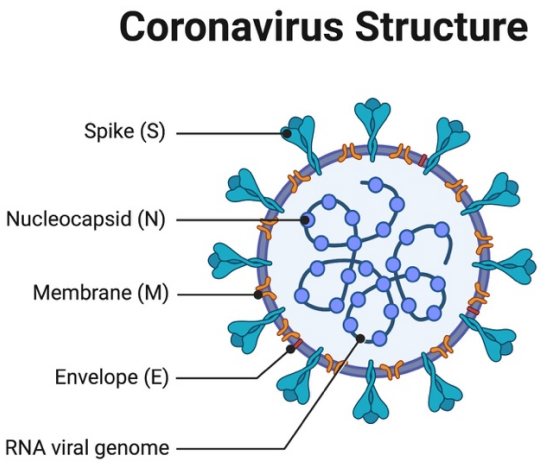

Figure 1: Structure of the Coronavirus – In this image you can clearly see the four structural proteins of COVID-19. The S protein on the exterior for receptor binding, the M protein and E protein surrounding the internal viral environment, and N proteins surrounding the RNA genome (Phelan, 2020).

While it is a respiratory disease, COVID-19 causes a wide range of symptoms that can affect other parts of the body such as the gastrointestinal, cardiovascular, and neurological systems. Sometimes, an infected individual is asymptomatic. Mild cases often have symptoms similar to the flu or the common cold such as fever, cough, chills, headache, nausea and vomiting, and tiredness. Interestingly, COVID-19 can also cause the loss of taste and smell for the host. Severe cases have the previous conditions and more severe symptoms such as hemoptysis, hypoxemia, difficulty breathing, and tachycardia. Another factor considered for the patients of COVID-19 is "long-haulers" which are individuals with symptoms that remain for weeks or months such as crackling or wheezing of the lungs and brain fog (Pustake, 2022). While severe cases could be detrimental to even the healthiest individuals, certain pre-existing medical conditions worsened the effects of SARS-CoV-2. For example, people suffering from cancer, liver disease, chronic lung diseases, diabetes type 1 or 2, heart conditions, and other

immunocompromised individuals are far more likely to suffer the extreme consequences of COVID-19 (“People with Certain Medical Conditions”).

With its vast range of symptoms and high rate of infection, SARS-CoV-2 quickly spread from China to around the world. Transmission of the virus is caused mainly by respiratory droplets expelled when an individual breaths or coughs. While often the breathing of these droplets by a non-infected individual is what causes the spread of the infection, droplets that are picked up off a surface followed by an individual touching their eyes, mouth, or nose can also cause infection. The long incubation period, two to fourteen days depending on which variant, highly contributed to the vast infection. During the incubation period, an infected individual could spread the disease before the symptoms have begun. Asymptomatic cases and mild cases also greatly contributed to the rapid spread of the disease (“If You've Been Exposed to the Coronavirus,” 2022).

Once symptoms are present or for precautionary measures, there are multiple ways to diagnose if someone is infected or has been infected by SARS-CoV-2. Looking for genetic evidence of the virus by reverse transcriptase polymerase chain reaction (RT-PCR) tests is the most accurate. Once a nasal or saliva sample is taken from the patient, the genetic material of the virus is selectively amplified numerous times in order for it to be detected. Unfortunately, these tests take several hours, require lab equipment often not found in a physician’s office, and are costly.

Antigen tests have become the most common way of distinguishing if COVID-19 has infected an individual. Like the RT-PCR testing, a physician collects a nasal or saliva sample. The sample is then mixed with a solution to break down the cells in the sample and placed on a strip with antibodies for the virus to determine if infection has occurred. Because of the ease and

quickness of the test, home kits allow for individuals to tests themselves without attending a physician. However, these tests do not provide the most accurate results because a large load of viral antigens in the sample is needed to produce a positive result which leads to early cases of COVID-19 going undiagnosed (Hafer, 2021). Another form of testing occurs following a patient's recovery. Antibody testing helps determine the number of infected people who have successfully recovered from the disease. By taking only a small sample of blood, a physician can determine if a patient has the necessary antibodies to combat future infections of the virus. While antibodies do not guarantee immunity, they have shown to lower the severity and duration of secondary infections (Marshall, 2022). However, the individual is still at risk of infection from a different variant of the virus despite these antibodies.

Treatment for SARS-CoV-2 varies on a case-by-cases basis. For most individuals that are healthy and have only mild symptoms, rest and over the counter medication such as Tylenol or Advil is recommended along with fluids to maintain hydration. For people with more severe cases, underlying conditions, or the elderly, more drastic measures may be required. Paxlovid, the leading antiviral medication, has shown that it can help, but treatment is very specific. Once the onset of symptoms occurs, time is limited for treatment to begin. Before treatment can begin, the physician must ensure that the patient does not have medication that could potentially cause side effects with the antiviral. Age must also be considered for the type of antiviral for treatment to avoid risky side effects in younger children ("Covid-19 Treatments and Medications," 2022).

Worldwide Effect

The COVID-19 pandemic took the world by storm and exposed the fragility of our social and economic systems. Despite nationwide shutdowns, border closures, and the quarantining of infected individuals, the virus rapidly spread from person-to-person and nation-to-nation. So far,

SARS-CoV-2 has led to the infection of 683,000,000 and the deaths of 6,829,000 worldwide. In America alone, it has infected over 106,000,000 and caused the deaths of just over 1,150,000 individuals as of March 2023 (“Coronavirus Cases”). While these numbers continue to grow, the effects of the original waves of COVID-19 can still be felt.

The greatest impact of COVID-19 came in the first year of its existence. Many nations shut down and stopped many means of production hoping to quell the rapid transmission. However, these shutdowns lasted longer than anticipated by many. Soon, the global workforce was facing extreme poverty because they were not able to work. Without the ability to work, they could not collect income to feed themselves or their families. Even if families had money saved or had the ability to maintain an income, food became difficult to find in grocery stores. Farmers greatly felt the impact of COVID-19 because they no longer had the ability to export their produce or import necessary materials to farm such as fertilizer, pesticides, and equipment (“Impact of Covid-19 on People's Livelihoods, Their Health and Our Food Systems”).

Without the ability to leave their home and lack of nutritious food, people soon began to suffer physically. Multiple studies have shown that the pandemic had a negative impact on the physical activity of people. Nations soon realized that a sedentary lifestyle during the pandemic could cause a drastic increase in serious cases of infection because of the risk associated with obesity and the virus. Promotions for spending time walking outdoors or performing indoor exercises were launched, but these attempts either did not spur motivation or did not create a large enough difference to offset the sedentary lifestyle. While it is noted that there was an increase in usage of walking trails and parks during the lockdowns, the usage of these locations was not available to all people (Park, 2022).

On top of physical decline, a vast majority of the world suffered from the effects of COVID-19 on their mental health. Humans by nature are social creatures going as far back as the start of humanity. Suddenly, people were no longer allowed to gather in groups and receive the face-to-face human interaction that is needed. Families were not able to travel to see one another because of nationwide constrictions, and in many places, groups could not meet in sizes of greater than ten. For a period of time, families were not able to attend to their loved ones perishing in hospitals. This lack of social interaction led many people to develop severe depression and anxiety. With the lack of physical interactions, humanity turned to social media in hopes of feeling connected. However, this produced no better outcome for mental health. Coverage of the virus, conspiracies that the virus did not truly exist, and predictions that humanity would become extinct flooded social media. With no certain answers to be found, people found themselves more uncertain of what outcome the virus would produce. This uncertainty for the future led many people to develop a deep fear for the virus and anxiety for what would become of them (Usher, 2020).

While adults dealt with the weight of the unknown, children's education also suffered greatly during this time. For example, children in the United States that were in kindergarten when the government issued the nationwide lockdown are now in the third grade. Over that time period, students either learned online, learned from their parents, or did not learn at all. Since their return, it is clear that no matter which form of learning they experienced, their learning has suffered greatly especially in the reading category. Many if not most students in the third grade are failing to read at the pre-pandemic national average. Since this learning usually takes place during the formative years that they were not in the classroom, teachers of these students are not prepared or conditioned to educate them on this necessary topic. Staggeringly, it is expected that

if a strong emphasis was placed on this age group now, it would take five or more years for them to reach their expected education pre-pandemic (Barshay,2022).

Chapter II: Current U.S. Vaccines

mRNA Vaccines

While it may seem that mRNA vaccines are a new technology to many, scientists have experimented with the use of mRNA in vaccinations for decades since its discovery in 1960. In as early as the 1990s, scientists tested with mRNA vaccines to see if immunity for influenza could be triggered in rats. Clinical trials in humans for the rabies and influenza virus have also been conducted (Beyrer, 2021). mRNA is essential for the production of proteins by cells. By encasing the mRNA proteins with lipid molecules, scientists have successfully delivered the mRNA strands into cells. Once inside of the cells, the cell's own mechanisms are used to produce viral proteins which are commonly surface proteins for the virus. The immune system is able to recognize this foreign protein and develop a response to destroy it ("What Are mRNA Vaccines and How Do They Work?" 2022). Because of the research of mRNA vaccines and the recent human trials with rabies and influenza, scientists looked to create a mRNA vaccine against the SARS-CoV-2 virus.

The first mRNA vaccine to gain approval from the Food and Drug Administration (FDA) was the v. Commonly referred to as the Pfizer vaccine or Comirnaty, this vaccine received emergency use authorization (EUA) for administration for ages 16 and older in December of 2020 and received full approval in August of 2021. By using mRNA material for the production of the S protein in COVID-19, the vaccine activates an immune response. The vaccine is given in two doses spaced out depending on age, three to eight weeks for individuals over the age of

twelve. Because the vaccine is not capable of granting long term immunity, booster shots are required to maintain the vaccines immunity ("FDA Approves First COVID-19 Vaccine," 2021). While Pfizer-BioNTech might have been the first to produce an approved vaccine, others companies were diligently working to produce a vaccination.

Almost immediately after the EUA for the Pfizer-BioNTech COVID-19 vaccine, Moderna received their EUA for their COVID-19 mRNA vaccine. Like Pfizer's vaccine, the Moderna vaccines use mRNA technology to spur the immune system into producing antibodies for the S protein of the COVID-19 disease (Cross, 2020). In terms of differences, Moderna and Pfizer both produced extremely similar vaccines to combat the pandemic. The mRNA sequences used by the companies differ only slightly in the portions of genetic code used to produce the S proteins. With similar time periods for the multiple shots required for vaccination and the requirement for boosters, these vaccines are nearly identical (Katella, 2023). While these vaccines have been vital for recovery from the COVID-19 pandemic, they are strikingly similar and not long-term solutions.

Johnson & Johnson

Only a few months following the approval of the mRNA vaccines, the FDA approved the distribution of the Johnson and Johnson (J&J) COVID-19 vaccine in February 2021. Unlike its predecessors, the J&J vaccine does not use mRNA to trigger the body's immune system. Instead, this vaccine uses a disabled adenovirus as a viral vector. This vaccine also carries the genetic information for the body to make COVID-19 S protein which the body will build immunity towards (Shae, 2021). Also, this vaccine only requires one original shot for the immune system to produce the antibodies necessary to compact the virus. However, booster shots of either Pfizer or Moderna are recommended to keep consistent immunity (Weber, 2021)

Novavax

The most recent FDA approval occurred for the Novavax vaccine in July of 2022. While it might have taken two years since the initial release of a COVID-19 vaccine in America, this vaccine circulated in several European countries for far longer. Unlike any of the previous vaccines, it directly injected the S protein of the SARS-CoV-2 virus via a nanoparticle protein subunit into the human body but cannot cause disease (Katella, 2023). The Novavax requires a two-dosage regimen to obtain immunity like the mRNA vaccines which is available for anyone over the age of twelve. A booster for the vaccine is available, but it is only approved for adults over the age of seventeen that have been made aware that they cannot receive a Pfizer-BioNTech or Moderna booster afterwards. Anyone between the ages of twelve and seventeen that have received a Novavax vaccine can receive either of the mRNA vaccines (Katella, 2022).

Current Vaccine Problems

There is no denying that the vaccines produced over the course of the pandemic have proved invaluable to making the steps in returning to normal life; however, they are far from perfect. Several of the problems associated with the current vaccines have gained large media and public attention. For example, both of the mRNA vaccines have become associated with myocarditis (inflammation of the heart) and pericarditis (inflammation of the outer lining of the heart). While these are considered rare side effects, they drew enough public attention and concern for the FDA to place a warning label on them. The Novavax vaccine was also associated with these forms of inflammation but at a much smaller scale in clinical trials. More FDA warning labels reside on the J&J vaccine. While rare, this vaccine became associated with a neurological disorder and a blood clotting disorder. The concerns for the association of the vaccine with these conditions grew to the point of placing a hold on this vaccine. After further

studies, the hold was lifted, but access to the vaccine is restricted (Katella, 2022). Another factor that has added hesitancy towards the vaccine is the relative newness of them. Although research for the concept of mRNA vaccines dates nearly sixty years, the two mRNA COVID-19 became the first ones to achieve approval by the FDA. This concept of newness and fear of long-term side effects has drastically impacted the push for herd immunity.

Herd immunity is achieved when an infected person is unable to contact enough susceptible people to spread the disease. In order to leave the susceptible pool, vaccinations and naturally immunity are common. However, rapidly mutating diseases, vaccines that are not 100% effective, and pockets of susceptible individuals greatly affect the herd immunity threshold (McDermott, 2021). Because the vaccines all require multiple booster shots to continue immunity, many are unwilling or unable to continually keep up to date with their vaccinations for SARS-CoV-2. With these two large groups not continuously maintaining immunity, it is unlikely that herd immunity can be reached (Mayo Clinic Staff, 2022). While these problems have caused quite the predicament, two problems loom over them.

Variants of SARS-CoV-2 have continuously evolved throughout the course of the pandemic. While several portions of the virus have mutated, the mutation of the S protein consistently mutates in each of the variants. Sometimes variants do not truly affect how the virus functions, but the variants of COVID-19 have proved to be of concern. In order to classify as a "variant of concern," the virus must have characteristics of a higher likelihood of infection, especially in subjects that have already contracted COVID-19 or received a vaccine. These variants often cause more cases of severe disease, resist antivirals, and do not trigger the immune system as if it were the original disease. With all four of the current vaccinations for the pandemic only containing information for the S protein, it is no surprise that the virus has

mutated specifically in this region. With the S protein constantly evolving, the vaccines and even boosters do not always have the ability to trigger the immune system to make the correct antibodies for the variants (Bollinger, et. al, 2022). While companies scramble to develop boosters for the vaccine, the virus will continue to mutate and infect millions of people.

Another pressing issue with the current vaccines is the inability to show long term immunity towards the virus. All of the different types of vaccines require an individual to get the main shot, whether it is two doses or a single one, followed by boosters to maintain immunity. With the increased amount of effort to continually maintain immunity, individuals consistently lose immunity to the virus (Katella,2023). This loss of immunity leaves individuals with the possibility of contracting the virus again, developing doubt in the scientific community's ability to produce a vaccine despite their own ignorance causing the increased risk of reinfection.

Chapter III: rVZV-SARS-CoV-2

Recombinant Vaccines

While relatively new to the scene, recombinant vaccines are making rapid strides in the medical field. There are three different subcategories to take into consideration for recombinant vaccines: DNA, mRNA, and protein. Despite their differences, the three types are all grown in a similar manner depending on genetic base pair size. The desired genetic sequence or sequences for antigen growth are removed and placed into a host genome such as bacteria, yeast, or virus. From there, the inserted genome grows with the host genome until it has replicated enough to be used in vaccinations (Nascimento, et. al, 2012).

rVZV-SARS-CoV-2

In order to provide a more long-term solution to the COVID-19 pandemic, new solutions needed to be created to fill the cracks in the current plan. A major way to set the world on the course would be a new vaccine that could lead to herd immunity. The rVZV-SARS-CoV-2 vaccine could be the solution.

This vaccine tackles the current vaccines' problems in multiple ways. By using a Bacterial Artificial Chromosome, the genetic codes for the N protein and the RBD protein were placed into the nonessential gene 13 of the VZV and grown in Escherichia coli (E. coli) cells. The insertion of both of the genetic sequences leads into the first advantage of this vaccine (Figure 2).

Figure 2: VZV Genome - VZV is composed of two regions. The Long (L) region which contains Unique Long (UL) components bracketed by Inverted Long (IRL) repeats, and the Short (S) region which contains Unique Short US components bracketed by Inverted Short (IRS) repeats. Either the SARS N or RBD genetic sequences are inserted into VZV Open Reading Frame (ORF) 13 in the rVZV-SARS-CoV-2 vaccines.

The rVZV-SARS-CoV-2 vaccines contains either the gene for the N antigen (rVZV-SARS-N) or a key part of the S antigen (rVZV-SARS-RBD) for the COVID-19 virus. With genetic code for both antigens present in a two-shot regimen, the chance that the virus mutates to form a variant declines significantly. Second, VZV is an optimal virus vector for public trust because a vast majority of children already receive the two shots for vaccination before the age of two. Adults also receive VZV vaccinations to combat shingles. Third, this vaccine is a live attenuated vaccine which means that unlike the current vaccines, the body has the ability to produce a strong, long-term immunity to the virus without the constant need for boosters. The combination of two key proteins of the virus and strong, long-term immunity with only two vaccinations potentially makes herd immunity achievable.

The VZV genome consists of 125,000 base pairs and 62 open reading frames. Gene 13 of VZV is considered nonessential because, when it is removed from the virus, replication is not affected making it the optimal location for inserting the genetic code for either the N or RBD antigen in either the recombinant VZV vaccine for the N antigen (rVZV-N) or recombinant VZV vaccine for the RBD antigen (rVZV-RBD).

Chapter IV: Methods

Infection of MeWo Cells with rVZV

The first step in research for rVZV-SARS-CoV-2 was seeing if it had the ability to infect cells. Human melanoma (Mewo) cells were chosen to infect in the laboratory because of their ability to rapidly grow and divide. Over the course of several days, cells were grown in 5% newborn calf serum (NBCS) until they were optimally sized for infection. Once the cell cultures had reached this point, cells were infected in three groups: rVZV-SARS-N, rVZV-SARS-RBD or wild-type varicella zoster virus (wtVZV), VZV without the genetic sequences for SARS to act as the control. These cells showed optimal infections across all three groups.

From here, the infected cells were processed for DNA analysis by polymerase chain reaction (PCR) and protein analysis by immunofluorescence and immunoblot as explained below.

DNA Analysis of rVZV by PCR for SARS N and RBD Genetic Sequences

PCR was used to determine if the SARS N and RBD genetic sequences were successfully placed into the VZV genome. DNA saved from the cell cultures went through many rounds of PCR testing separated into three categories: rVZV-SARS-CoV-2 N DNA tested with nucleocapsid primers, rVZV-SARS-CoV-2 RBD DNA tested with RBD primers, and wtVZV DNA tested with each of the types of primers. In order to perform these experiments, the extracted DNA was mixed with the following: primers specific to the desired region for amplification, distilled water (dH_2O), and PCR BIO 2x Taq DNA Polymerase for creating the

new strands of DNA. After the mixtures were completed, they were placed in a thermocycler to undergo the PCR steps: denaturing of the DNA, annealing of the primers to the DNA, and elongation of the new DNA strand. PCR conditions were broken into three blocks: 95º C for 120 seconds for original denature; thirty repeats of a 95º C denature for 20 seconds, 57º C annealing for 60 seconds, and 72º C extension for 60 seconds; and a 4º C infinite time until removed from thermocycler. Once fully prepared, dye was added to the mixture before being inserted into individual wells of a 0.5% Agarose gel for gel electrophoresis. In the gel, the negatively charged DNA was pulled down the gel via positive charge. The larger strands of base pairs will travel a shorter distance from the wells due to size constriction while smaller strands travel farther. Because the base pair size of the amplified regions is known, it can be confirmed whether the correct DNA section was amplified when imaged. For the first PCR experiment, RBD-primers 4289 and 4290 which amplify an expected 302 base pair regions, and N-primers 1711 and 1712 which amplify an expected 148 base pair regions were utilized. After the first PCR, a different set of N primers, 1713 and 1712, and RBD primers, 4287 and 4290, were ordered to achieve a secondary confirmation testing (Table 1).

Primers	Expected Base Pair Size
VZV-RBD Primers 4289 and 4290	302
VZV-RBD Primers 4287 and 4298	576
VZV-N Primers 1711 and 1712	148
VZV-N Primers 1713 and 1712	1106

Table 1: rVZV Primers and Base Pair Sizes – This table represents the sizes for the genetic region that each of the primers will amplify which allows for confirmation by location on a gel.

DNA Analysis of rVZV by Nested PCR for Gene Location of SARS N and RBD Sequences

To conclude the DNA analysis of rVZV-SARS-CoV-2, a nested set PCR was used to determine that the genetic code for the N or RBD antigens was correctly placed in gene 13 of each rVZV vaccine (Figure 2). A nested set PCR allows for the precise location of a genetic sequence by breaking a normal PCR process into two sections. The first PCR conducted used primers for VZV gene 13, which would be present in the recombinant VZV (rVZV) DNA and wtVZV DNA. The primers utilized were Gene 13 FWD EXT 6996 and Gene 13 REV EXT 6997 which produce an expected band of 1153 base pairs. Aside from using a common primer for rVZV-N, rVZV-RBD, and wtVZV, all other steps and conditions for the nested set PCR step one were the same as previous PCRs. Following these steps, the vials that contain the amplified DNA genome were utilized as the DNA for the second step of the PCR. The conditions for the second step were split into three blocks: 95º C for 120 seconds for original denature; thirty repeats of a 95º C denature for 15 seconds, 57º C annealing for 60 seconds, and 72º C extension for 30 seconds; and a 4º C infinite time until removed from thermocycler. Depending on which amplified region was used, the amplified rVZV-RBD and rVZV-N were mixed with either RBD primers 4287 and 4290 or N primers 1711 and 1712 (Table 1). The wtVZV vaccine was mixed with both sets of primers.

Immunoblot Assay for SARS N and RBD Antigens in rVZV Infected Cells

In order to determine if the cells infected with rVZV-SARS-CoV-2 expressed N and RBD antigens from the inserted genetic sequences, an immunoblotting technique was used. An immunoblot, also known as a western blot, separates and identifies proteins inside of a cell. In order to accomplish this, the protein complexes of the infected cells from the rVZV and the wtVZV cultures were mixed with Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) buffer which gives the protein a uniform charge so that the only physical

characteristic that differentiates the proteins is their size. Next, the uniform protein was separated by gel electrophoresis with larger proteins coming to rest at the top and smaller traveling farther down the gel. After the gel electrophoresis, the proteins were transferred to a membrane via electrophoretic transfer for better handling. Once the proteins transfer, a scalpel cuts the membrane into individual strips to place in a container with individual well chambers for each strip. The strips mirror the gel consisting of: molecular weight marker lane, two control lanes from the wtVZV, rVZV-RBD lane, and rVZV-N lane. In their chambers, the strips were washed several times with BLOTTO, a solution containing dried milk that attaches to nonspecific binding sites to prevent antibody attachment.

To begin the antigen testing, a primary antibody, either rabbit anti-SARS-2 N or rabbit anti-SARS-2 RBD produced by Sino Biological in Beijing, China, was added to the chamber wells. One control receives the antibody for N and the other receives the RBD antibody; the remaining strips were coated in their respective antibody for which rVZV vaccine the cells were infected with. Another control experiment consists of each rVZV infected cells being washed with normal rabbit serum. These controls were used to show that rabbits do not have natural antibodies to the SARS antigens.

The well chambers were placed in an incubator for three hours with constant movement to ensure that the antibodies can adequately attach to the antigens. The chamber was removed and strips washed with a buffer solution. A secondary antibody, goat anti-rabbit IgG-peroxidase produced by Fisher Scientific, was placed onto all strips which undergo the same rocking for a similar timespan. The peroxidase portion of the secondary antibody serves the crucial purpose of holding an enzyme substrate on the end of the antibody. In order to activate this enzyme, the strips were bathed in a chemiluminescence reagent from Pierce immediately before imaging by

the Bio-Rad Chemidoc Imager. For these images, the expected size for the expressed protein in each vaccine is 30 kilodalton (kD) for RBD and 45 kD for N.

Immunofluorescence Assay for SARS N and RBD Antigens in rVZV Infected Cells

In order to confirm the presence of antigens within rVZV-N and rVZV-RBD infected cells, an immunofluorescence assay was performed. To begin this experiment, the concentration of the infected cells must first be counted on a hemocytometer to determine the proper amount of solution needed to fix the cells to chamber wells for imagining. Once the ratio was determined, the cells were fixed in culture slide chamber wells via paraformaldehyde. Triton was then used to make cells permeable to antibodies. The chamber wells were split into rVZV-N, rVZV-RBD, and wtVZV. After the cells were fixed and permeable, they were washed with an immunofluorescence blocking buffer to prevent non-specific binding of the primary antibody.

Once the cells were fully prepared, the wells were covered in antibody solutions. Like the immunoblot experiment, the primary antibodies used for the immunofluorescence were rabbit anti-SARS-2 N or rabbit anti-SARS-2 RBD for the respective chamber wells. These antibodies were produced by the same company as the antibodies utilized in the immunoblot assay. The wtVZV sample was used in two chamber wells so it could be washed with both of the rabbit antibodies. After an incubation period to allow the primary antibodies to attach to the antigens, the secondary antibody was applied. Once again, a goat anti-rabbit antibody was used from Fisher Scientific. However, this antibody contains a specific fluorescent dye that allows us to see whether the cell has expressed antigens for the primary antibody to attach. Once the secondary antibody was applied, the wells were quickly washed to remove any unbound antibodies before the fluorescent images were taken on a confocal microscope.

Chapter V: Results

PCR DNA Analysis to Confirm SARS RBD and N Genetic Sequences in rVZV Vaccines

PCR was used to confirm the presence of SARS N and RBD genetic sequences in the rVZV-SARS-CoV-2 vaccines. In the imaging for the PCRs of rVZV-N and rVZV-RBD, bands with the correct genetic size for the sequences need to be present. With bands present, it can be concluded that the rVZV-SARS-CoV-2 vaccines contain the necessary genetic code to provide immunity to COVID-19.

The first PCR utilized rVZV-N primers 1711 and 1712 for N with an expected size of 148 base pairs and rVZV-RBD primers 4289 and 4290 with an expected size of 302 base pairs (Table 1). Genetic code for N in the rVZV-N vaccine and genetic code for RBD in the rVZV-RBD vaccine were shown on the gel with the corresponding base pair size while no bands were detected for wtVZV. While proving the presence of rVZV-RBD and rVZV-N genetic regions for their antigens, the experiment also showed no bands for wtVZV (Figure 3).

In the secondary experiment to confirm the results of the first PCR, N primers 1713 and 1712 for were used in rVZV-N for a 1106 base pair size, and RBD primers 4287 and 4290 were used in rVZV-RBD for a 576 base pair size (Table 1). This experiment confirmed the results obtained in the first PCR. However, there is an unknown binding region creating a band for the N primer for the rVZV-N vaccine (Figure 4).

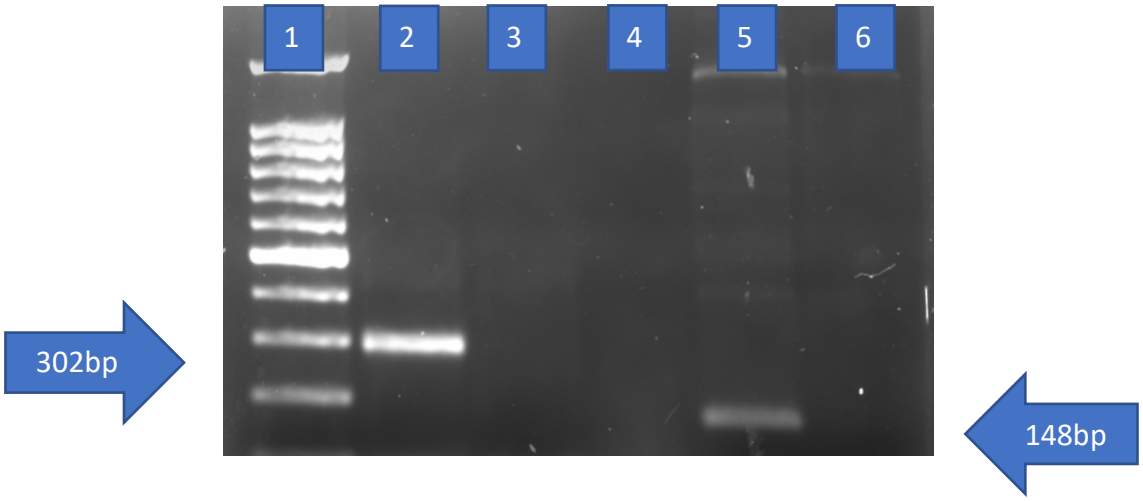

Figure 3: PCR Confirms Presence of SARS N and RBD Genetic Sequence in rVZV-SARS Vaccine – Lanes: (1) Molecular Weight Marker (MWM), (2) VZV-RBD with RBD primers 4289 and 4290, (3) wtVZV with RBD primers 4289 and 4290, (4) Empty, (5) VZV-N with N primers 1711 and 1712, (6) wtVZV with N primers 1711 and 1712

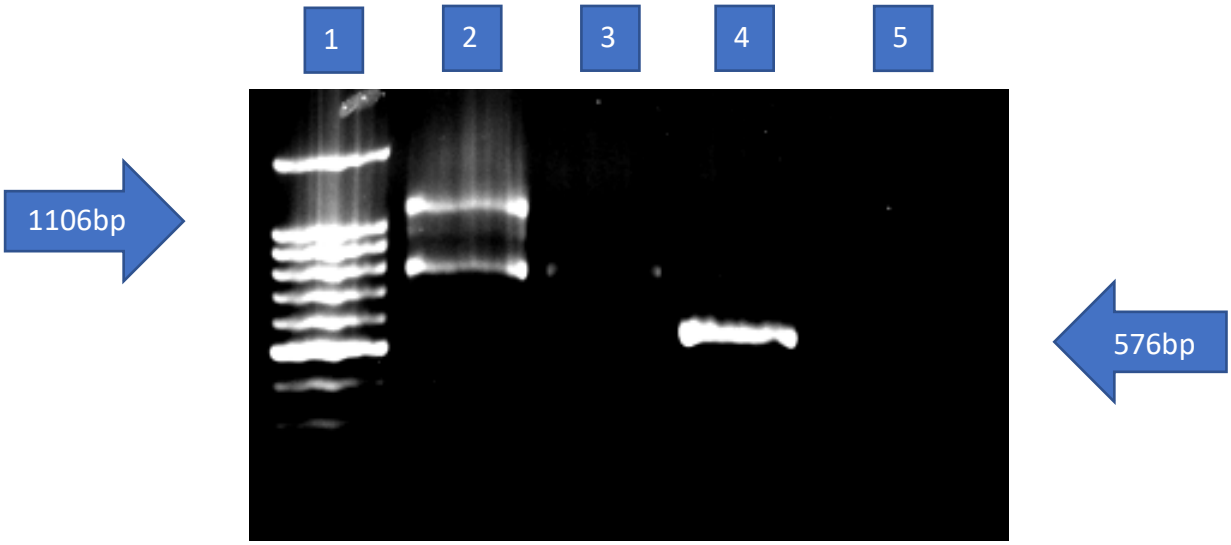

Figure 4: PCR Confirms Presence of SARS N and RBD Genetic Sequences in rVZV-SARS Vaccine for Secondary Confirmation – Lanes: (1) MWM, (2) VZV-N with primers 1713 and 1712, (3) wtVZV with primers 1713 and 1712, (4) VZV-RBD with primers 4287 and 4290, (5) wtVZV with primers 4287 and 4290

Nested Set PCR DNA Analysis to Confirm SARS N and RBD Genetic Sequences in rVZV Vaccine ORF 13

A nested set PCR was performed to show that the SARS RBD and N genetic sequences were successfully inserted into rVZV vaccine ORF 13. This step is necessary to determine if the

virus vector can still grow and reproduce along with the inserted genetic sequence. For rVZV-SARS-CoV-2, gene 13 is the targeted gene because it is not essential gene for VZV replication. The first step of the experiment successfully showed that gene 13 is present in wtVZV, rVZV-N, and rVZV-RBD at the expected 1153 base pair region (Figure 5).

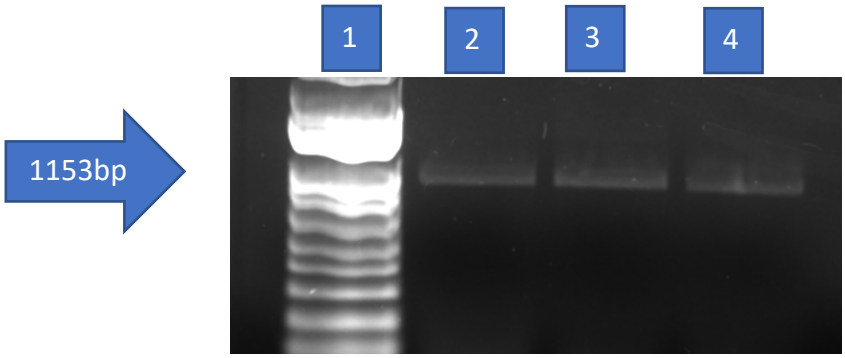

Figure 5: Nested Set PCR of rVZV and wtVZV for Gene 13 – Lanes: (1) MWM, (2) VZV-N, (3) VZV-RBD, (4) wtVZV.

Using the amplified gene 13 region of wtVZV, rVZV-N, and rVZV-RBD, the second step to determine if the genetic code for N and RBD proteins are present in gene 13 can be performed. If the genetic information observed in Figure 3 and Figure 4 is shown, then the rVZV-N and rVZV-RBD genetic sequences are in the correct placement for their vaccines.

This experiment successfully showed the presence of N antigen genetic code in the rVZV-N vaccine and RBD antigen genetic code in the rVZV-RBD vaccine at the correct band size location within gene 13 of VZV. No genetic code was triggered for either the N or RBD primer in the wtVZV vaccine.

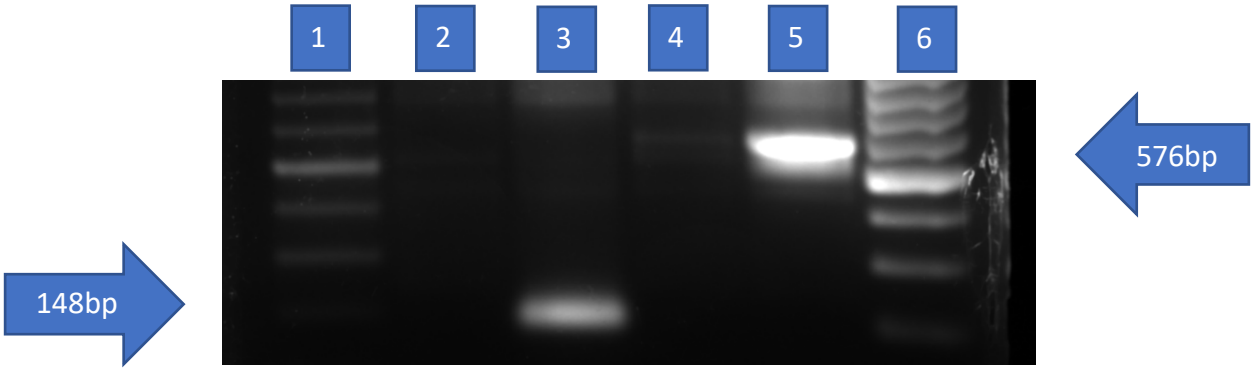

Figure 6: Nested Set PCR Confirms SARS N and RBD are in Gene 13 of rVZV-SARS Vaccine – Lanes: (1) Promega 100bp MWM 14µL, (2) wtVZV 10 µL with primers 1711 and 1712, (3) VZV-N 15µL with primers 1711 and 1712, (4) wtVZV 10µL with primers 4287 and 4290, (5) VZV-RBD 15µL with primers 4287 and 4290, (6) Generuler 100bp MWM 4µL

Immunoblot Protein Analysis Confirming Production of SARS N and RBD Antigens in rVZV Infected Cells

Immunoblot analysis was performed to confirm that rVZV infected cells produce SARS RBD and N proteins. In this experiment, we expected to see bands for the rVZV-N N protein and rVZV-RBD RBD protein at the correct size marker, 45kD for N and 30kD for RBD.

For the first set of images, the band for the N antigen at 45kD of the SARS virus is expressed in the rVZV-N infected cells (Figure 7a). The first control set focused on the N antigen. The wtVZV infected cells were washed with rabbit anti-SARS N (Figure 7b), and another set of rVZV-N infected cells was washed with normal rabbit serum (Figure 7C). Because neither control for the N antigen expressed a band, it can be concluded that the band for rVZV-N washed with rabbit anti-SARS N is specific to the N antigen (Figure 7).

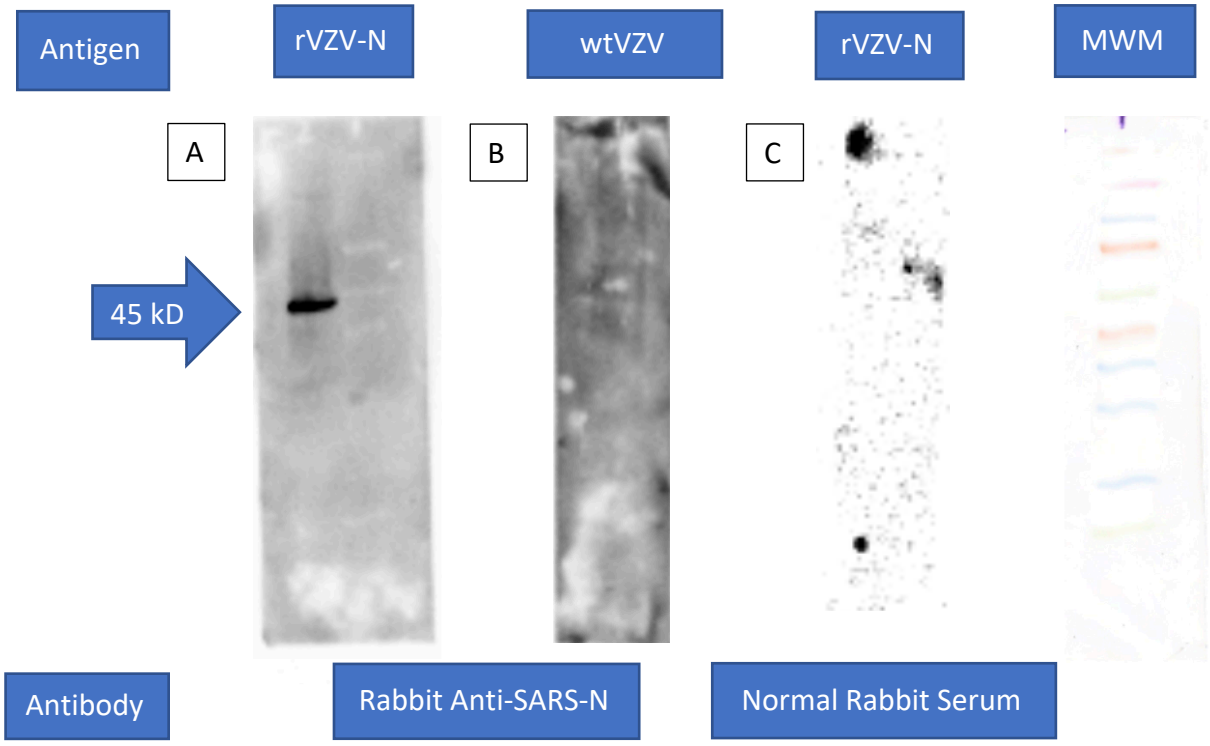

Figure 7: Immunoblot Confirms Cells Infected with rVZV-SARS N Produce Specific SARS N Antigen – Pictured left to right: rVZV-N with rabbit anti-SARS-2 N, wtVZV-N with rabbit anti-SARS-2 N, rVZV-N with normal rabbit serum, and a MWM lane.

The second set of images for the immunoblot assay confirm rVZV-RBD infected cells express the SARS RBD antigen at 30kD. A second band on the rVZV-RBD immunoblot assay was attributed to the breakdown of product (Figure 8a). The second control set focused on the RBD antigen. The wtVZV infected cells were washed with rabbit anti-SARS RBD (Figure 8b), and another set of rVZV-RBD infected cells was washed with normal rabbit serum (Figure 8c). Because neither control for the RBD antigen expressed a band, it can be concluded that the band for rVZV-RBD washed with rabbit anti-SARS N is specific to the RBD antigen (Figure 8).

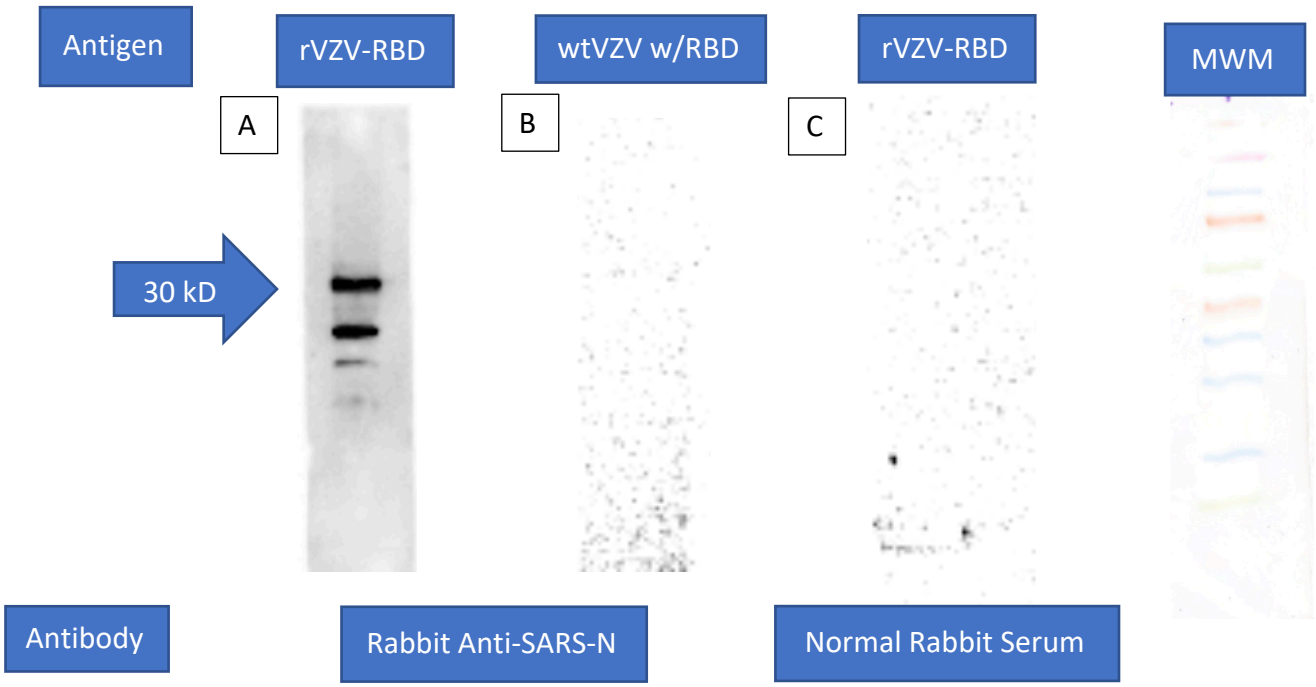

Figure 8: Immunoblot Confirms Cells Infected with rVZV-SARS-RBD Produce Specific SARS RBD Antigen – Pictured left to right: rVZV-RBD with rabbit anti-SARS RBD, wtVZV with rabbit anti-SARS-2 RBD, rVZV-RBD with normal rabbit serum, and MWM.

Immunofluorescence Assay to Confirm SARS RBD and N Protein Expression in rVZV Infected Cells

Immunofluorescence was used to further confirm that rVZV infected cells are producing and RBD and N antigens. For the rVZV-N infected cells, it can be clearly seen that antibodies for the N antigen were able to attach. Therefore, it can be concluded that rVZV-N infected cells express SARS N antigens internally (Figure 9a). For the control, wtVZV infected cells washed with antibodies for the N antigen showed no sign of attachment (Figure 9b). The exclusive binding in the rVZV-N infected cells confirms that SARS-N antigens are specific to the rVZV-N vaccine. (Figure 9).

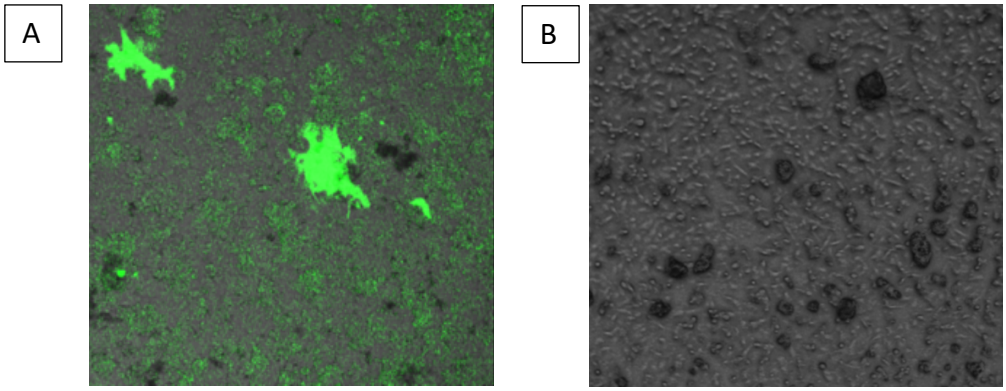

Figure 9: Immunofluorescence Confirms That rVZV-SARS-N Infected Cells Contain the SARS-2-N Antigen and Control Immunofluorescence of wtVZV Infected Cells Has No SARS-2-N Antigen

Cells infected with rVZV-RBD showed clear signs of internal SARS RBD antigens based on the ability of antibodies for the RBD antigen to attach (Figure 10a). The wtVZV control for RBD antigens showed no sign of antibody attachment (Figure 10b). It can be concluded that cells infected with rVZV-RBD have specific internal SARS N antigens from the rVZV-RBD vaccine (Figure 10).

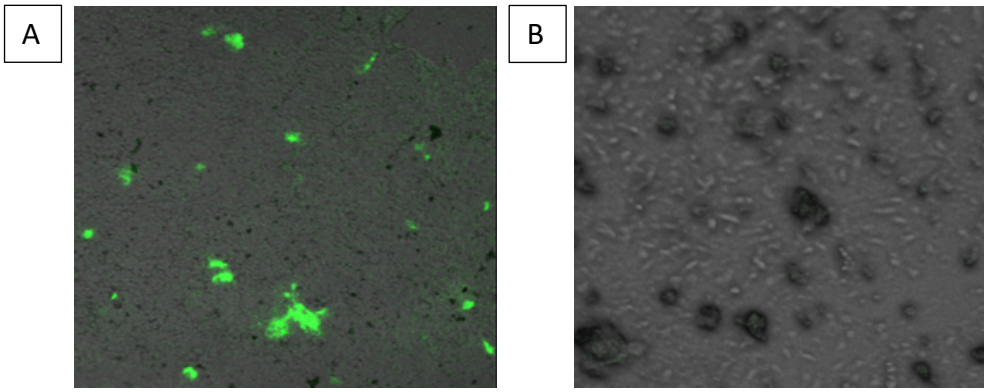

Figure 10: Immunofluorescence Confirms That rVZV-SARS-RBD Infected Cells Contain the SARS-2-RBD Antigen and Control Immunofluorescence of wtVZV Infected Cells Has No SARS-2-RBD Antigen

Chapter VI: Discussion

The results gathered from this research strongly support the success in the development of the rVZV-SARS-CoV-2 vaccines. The PCR studies proved that the genetic code implemented in the rVZV-N vaccine for the N antigen and the genetic code implemented in the rVZV-RBD vaccine for the RBD vaccine are present in (Figure 3, 4). The nested set PCR showed that these genetic codes are successfully placed in gene 13 (Figure 5, 6). With the genetic code present and successfully placed in this region, the vaccines carry the necessary genetic information for cellular production of the SARS-CoV-2 antigens and do not affect VZV vaccination since gene 13 is not necessary for viral infection. When testing cells for their ability to produce the N and RBD antigens, two studies successfully showed production. In the immunoblot studies, lysates of infected cells with the rVZV-N vaccination confirmed presence of N antigens (Figure 7). The rVZV-RBD confirmed the presence of RBD antigens at the correct size margin (Figure 8). Control immunoblots for wtVZV with both sets of antibodies and immunoblots in which normal rabbit serum were used showed negative results which proves that the bands present in rVZV-N and rVZV-RBD are specific (Figure 7,8). The presence of proteins in vaccinated cells were once again proved by immunofluorescence assays. When imaged, attachment by the primary and secondary antibodies to the antigens are clearly seen (Figure 9, Figure 10). With these results, it is confirmed that rVZV-SARS-CoV-2 vaccines express SARS RBD and N antigens.

Chapter VII: Conclusion and Future Research

The COVID-19 pandemic has substantially impacted the world in a multitude of ways. It infected over half a billion people and led to the deaths of millions. Travel restrictions, quarantines, sedentary lifestyles, and lack of social interactions caused a drastic increase in depression and anxiety across the world. Although current vaccines have been of utmost value in recovering from the initial impact of the virus, they are far from perfect. The utilization of only one of the SARS-CoV-2 antigens led to many different forms of variations due to the virus's ability to rapidly adapt. In addition to the variations, current vaccines do not provide long term immunity for the patient, leading to the need of boosters regularly to maintain immunity.

With the development of the rVZV-SARS-CoV-2 vaccine, the problems of current vaccines can be resolved. Because it is a live vaccine, strong, long-term antibodies are produced. With these antibodies, there is no need to continuously receive boosters to maintain immunity. Also, the two-shot regimen uses genetic coding for N and RBD antigens instead of only the S antigen. By using genetic coding for two parts of the virus instead of one, the virus is less likely to mutate into variants that can avoid the immune system's antigens. With these two critical problems solved, herd immunity can potentially be reached. With proper distribution and compliance, the pool size for immune individuals will increase while the pool for susceptible individuals will decrease which will lead to fewer infections.

The success of this research for the development of the rVZV-SARS-CoV-2 vaccine will lead to future research. The next steps in research will be conducted on various animals such as

mice to see if immunity to COVID-19 is achievable. If there is success, research will move on to monkey models for a more accurate, comparable measurement for humans. Immunity achieved in these studies will lead to clinical trials where the vaccine will undergo the ultimate tests of preventing infection of SARS-CoV-2 in humans.

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