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Characterization of a Plasmid-Based DNA Vaccine for Simian Immunodeficiency Virus

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CHARACTERIZATION OF A PLASMID-BASED DNA VACCINE FOR SIMIAN IMMUNODEFICIENCY VIRUS

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

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DEDICATION

I would like to dedicate this thesis to my parents. I am especially grateful for their unwavering support and encouragement throughout my four years at The University of Mississippi. Thank you for always being there for me.

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I would first like to thank Dr. Wayne L. Gray and The University of Mississippi Biology Department for providing me the opportunity to work on this project. I would like to extend my thanks to Dr. Gray for allowing me to work in the virology lab and expand my knowledge of vaccine development. I have gained a new appreciation for the numerous hours of hard work and patience that is involved in developing a vaccine. I would also like to thank my thesis committee, Dr. Brian Doctor and Dr. Gregg Roman, for taking the time to read and edit my thesis.

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Lastly, I would like to extend by gratitude towards the Sally McDonnell Barksdale Honors College for providing various opportunities to enrich my academic experience throughout these four years at the University of Mississippi.

The Retanef gene sequence used in this research was created by Dr. Genoveffa Franchini.

ABSTRACT

PRIYA CHETAN SANIPARA: Characterization of a Plasmid-Based DNA Vaccine for Simian Immunodeficiency Virus (Under the direction of Dr. Wayne L. Gray)

Described as one of the world's worst pandemics, HIV (Human Immunodeficiency Virus) infects millions of people each year and is the cause for AIDS (Acquired Immunodeficiency Syndrome). Despite the development of vaccines for numerous infectious diseases such as polio, small pox, and influenza, a vaccine for HIV remains elusive due to the virus's high mutation rate and ability to evade the immune system. HIV causes depletion of CD4+ lymphocytes, resulting in a weakened immune system. However, the development of a plasmid-based DNA vaccine approach may help revolutionize vaccine development for HIV due to its ability to confer cellular and humoral immunity through T-cells and antibodies, respectively. The already constructed pVAX1 plasmid's small size and multiple cloning sites make it an effective vector for the development of a plasmid-based DNA vaccine for HIV. In this experiment, a gene sequence for SIV (Simian Immunodeficiency Virus) proteins inserted into the pVAX1 plasmid was transfected into an African green monkey kidney cell line (Vero cells) for expression of SIV proteins.

HIV and AIDS research aims to contribute to the development of prevention and treatment strategies for this disease. The research in this thesis focuses on characterization of a plasmid-based DNA vaccine expressing *rev*, *tat*, and *nef* (Retanef) proteins for SIV using restriction endonuclease analysis, PCR, immunofluorescence, and

western blotting. The specific aims for this thesis include: evaluating the presence of the Retanef gene sequence inside pVAX-RTN, and analyzing the expression of the Retanef protein in vitro. This study showed that transfection of pVAX-RTN into Vero cells resulted in successful expression of the Retanef protein. Detection of the Retanef protein by SIV positive monkey sera also showed positive results, further proving that Retanef is expressed inside Vero cells via the pVAX-RTN plasmid.

The results acquired in this experiment will contribute towards HIV vaccine research by using SIV as the experimental model for HIV. The results acquired in this research supports the use of early regulatory proteins in SIV vaccines and will be contribute towards successfully creating an effective HIV vaccine in the future.

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LIST OF ABBREVIATIONS

1°	Primary
2°	Secondary
AIDS	Acquired Immunodeficiency Syndrome
BSA	Bovine Serum Albumin
CO_2	Carbon Dioxide
CMV	Cytomegalovirus
DAPI	4′,6-diamidino-2-phenylindole
EBV	Epstein Barr Virus
FITC	fluorescein isothiocyanate
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus Type 1
HIV-2	Human Immunodeficiency Virus Type 2
kDa	Kilodalton
MHC	Major Histocompatibility Complex
μg	Microgram
μL	Microliter
NIH	National Institutes of Health
PBS	Phosphate Buffered Saline
PCP	*Pneumocystis carinii* pneumonia
PCR	Polymerase Chain Reaction
PVDF	Polyvinylidene fluoride
RTN	Retanef
RT-PCR	Reverse Transcription Polymerase Chain Reaction

SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SIV	Simian Immunodeficiency Virus
SIV_{cpz}	Chimpanzee Strain of SIV
SIV_{gor}	Gorilla Strain of SIV
SIV_{gsn}	Greater-spot Nosed Monkey Strain of SIV
SIVmac251	Macaque Strain of SIV
SIV_{rcm}	Red-capped Mangabey Strain of SIV
SIV_{smm}	Sooty Mangabeys Strain of SIV

INTRODUCTION

Throughout history, ever since the first humans spread across the world, outbreaks of infectious disease have been common. Only a few of these outbreaks have been able to reach a pandemic level, such as smallpox, bubonic plague, and the Spanish flu. As scientists developed a better understanding of disease transmission and mechanism of action, many of these diseases were eradicated or prevented from occurring again by means of proper sanitation, prevention, and treatment. Over the years, the development of vaccines has better prepared us to face diseases and the challenges they bring. However, creating a vaccine involves an incredible amount of time and effort, and new diseases are accompanied with their own unique challenges. Discovered in the 1980s, HIV (Human Immunodeficiency Virus) has become a pandemic, infecting millions of people around the world. Without treatment, HIV weakens the immune system and progresses to AIDS (Acquired Immunodeficiency Syndrome), which leaves the affected individual vulnerable to opportunistic infections. Effective treatments for HIV only maintain the viral particles at a manageable level; the affected individual has to live with HIV for the rest of their life. The development of a vaccine for HIV is crucial to slowing the spread of this disease and preventing new infections. The research conducted in this thesis will contribute towards vaccine development in the global fight against HIV/AIDS.

This project uses SIV (Simian Immunodeficiency Virus) as a model for HIV for the characterization of a plasmid-based DNA vaccine. A previously constructed plasmid

containing the gene sequence for SIV early regulatory (*rev* and *tat*) and accessory (*nef*) proteins was analyzed for expression in African green monkey kidney cells (Vero cells). The features of the vaccine were analyzed using various lab techniques as follows:

- Restriction endonuclease analysis was used to determine the size of the Retanef gene sequence within the plasmid.

- PCR and gel electrophoresis was used to determine the orientation of the Retanef gene sequence within the plasmid.

- Immunofluorescence was used to determine the expression and localization of the desired Retanef protein within the transfected Vero cells.

- Western Blotting was used to identify the protein within the transfected Vero cells.

Characterizing this vaccine and evaluating the expression of the desired antigen within Vero cells will contribute towards the possibility of using this vaccine in trials using non-human primate models.

CHAPTER 1: BACKGROUND ON HIV/AIDS

In 1981, the Centers for Disease Control and Prevention's Morbidity and Mortality Weekly report first described Acquired Immunodeficiency Syndrome (AIDS) in five homosexual men diagnosed with an opportunistic infection of *Pneumocystis carinii* pneumonia in California (De Cock et al., 2011). The disease was also later identified in heterosexual individuals receiving blood products and babies born to infected mothers (Piot & Quinn, 2013). Although the causative agent was yet to be identified, preventative methods based on risk groups and modes of transmission were already being implemented. Since the discovery of the Human Immunodeficiency Virus (HIV) in 1983 as being the etiologic agent for AIDS, public health organizations have made many advances in treatment, screening, and prevention of AIDS. Despite these efforts, a vaccine for HIV is not yet available, and AIDS remains one of the world's worst pandemics (De Cock et al., 2011).

As of 2018, there were 37.9 million people in the world living with AIDS, with an average of 1.7 million new infections occurring each year. However, only 21% of those individuals had access to HIV testing, and only 62% had access to antiretroviral therapy. The majority of individuals affected by this disease are from low or middle-income countries that do not have access to proper HIV prevention and treatment. HIV testing and treatment are crucial to preventing the progression to AIDS and ending transmission of the virus. Despite these setbacks, AIDS-related deaths had decreased from 1.2 million

in 2010 to 770,000 in 2018. With more efforts to increase access in resource-poor countries and investment in HIV/AIDS research by the NIH, more progress is being made towards more effective treatments and a cure ("Global HIV/AIDS Overview").

Origin and Emergence of HIV/AIDS

AIDS is caused by the human immunodeficiency virus (HIV), a Lentivirus in the family Retroviridae that is closely related to simian immunodeficiency virus (SIV). There are two types of HIV (HIV-1, HIV-2), and each one is thought to have originated via cross-species infection of a simian precursor from non-human primates to humans. The African green monkey was the first species to be found infected with SIV. Non-pathogenic to the host species, SIV primarily infects monkeys and is species-specific (Sharp & Hahn, 2011). However, the virus is pathogenic if it infects a non-host species. For example, chimpanzees may have acquired SIV_{cpz} via a recombination event resulting from cross-species transmissions of SIVs by two different monkey species. Greater spot-nosed monkeys are the natural reservoir for SIV_{gsn}, and red-capped mangabeys are the natural reservoir for SIV_{rcm}. Chimpanzees may have acquired these two SIVs by predation which resulted in the two viruses recombining to form a virus capable of also infecting humans, SIV_{cpz}. This virus then evolved to HIV-1 as a result of host adaptation. Although similar in structure, HIV-1 and HIV-2 differ in their evolutionary pathways and pathogenicity. HIV-2 is less pathogenic and is closely related to SIV_{smm}, a virus naturally infecting West African sooty mangabeys. In a similar cross-species transmission event, HIV-2 is thought to have originated by humans getting infected with SIV_{smm}, and the virus adapting to the host to form HIV-2 (Sharp & Hahn, 2010). Surprisingly, there is

also evidence of cross-species transmission of SIV between chimpanzees and gorillas, resulting in SIV_{gor}. Scientists have yet to discover how this virus was transmitted because gorillas are herbivores (Sharp & Hahn, 2010).

Additionally, each type of HIV has multiple sub-types resulting from independent cross-species transmission events. The sub-types of HIV-1 include group M, N, O, and P, with M being the pandemic form and accounting for the majority of AIDS cases. Group O and N are less prevalent than group M, and infections involving these subtypes are mainly found in Cameroon. Group P is even less prevalent; the virus was only found in two individuals from Cameroon. Less likely to progress to AIDS and with lower transmission rates, HIV-2 consists of groups A and B (Sharp & Hahn, 2011).

HIV Viral Transmission

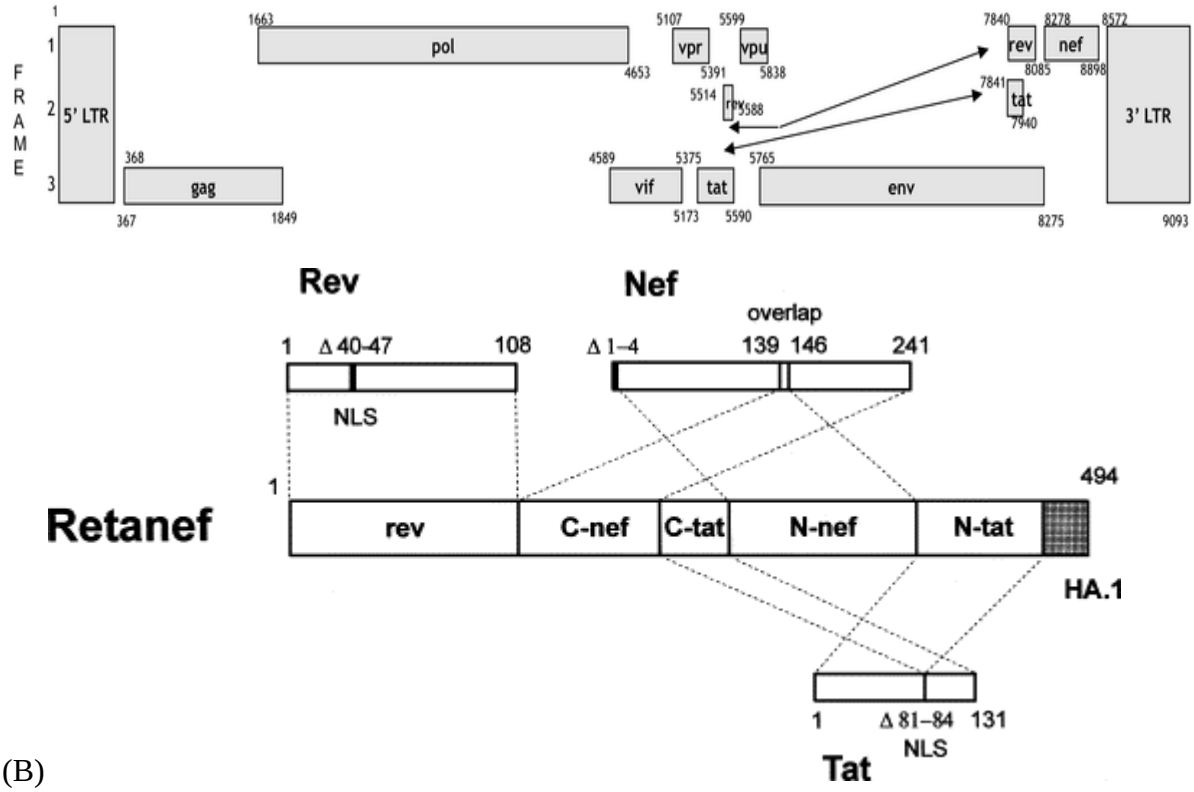
HIV is primarily transmitted through exposure to infected bodily fluids or blood via injured skin or mucosa. This can be through blood transfusions, organ transplants, sexual intercourse, breastmilk, childbirth, or contaminated needles (German Advisory Committee Blood, 2016). However, since the majority of HIV cases are the result of transmission via sexual intercourse, HIV is classified as a sexually transmitted disease (STD) (Sharp & Hahn 2011). Although less common, HIV is also transmissible by contact with an infected person's saliva to open legions and bite injuries. The chances of infection increase if the infected individual's virus titers are higher. Additionally, pregnant women can transmit the infection to the baby during the last trimester or earlier, depending on viral titers. Although HIV could be considered a zoonotic disease in the early part of the 20th century, it is currently only transmissible between humans.

Therefore, HIV is not transmitted via animal vectors; it is also not spread by food or water (German Advisory Committee Blood, 2016).

HIV Viral Structure

HIV has many key proteins that assist in viral entry, and it is important to understand their function before discussing pathogenesis. HIV is 100 nm in diameter and is composed of an outer lipid bilayer, which functions as the envelope and assists the viral particle with entry into the host cell. The envelope of the virus contains spikes formed by trimers of a surface protein (gp120), which are attached to the membrane by trimers of a transmembrane protein (gp41). The inner viral capsid is composed of a capsid protein (p24), and the outer capsid membrane is assembled by a matrix protein (p17). The core of the virus contains two single-stranded RNA molecules with enzymes that assist in viral replication. Additional regulatory and accessory proteins such as rev, tat, nef, vif, vpr, and vpu contribute to viral production and pathogenicity (German Advisory Committee Blood, 2016). The HIV genome and structure of SIV-Retanef is shown in Figure 1.

(A)



(B)

Figure 1 – Structure of SIV genome and Retanef Sequence - (A) – Structure of SIV Genome – The structure and organization of reading frames encoding the genes for the various structural and regulatory proteins. Notice that the *rev* and *tat* are composed of two gene regions. **(B) - Structure of RTN sequence -** Schematic representation of the chimeric Retanef protein. C: carboxy terminus of the protein; N: amino terminus. The sequences for *rev* and *tat* were separated and rearranged to disrupt the functionality of these two proteins. A partial sequence for *tat* was inserted between the two separated *nef* sequences to prevent *nef* from rearranging itself back to its pathogenic form. Figure A is adapted from, "New Strain of Simian Immunodeficiency Virus Identified in Wild-Born Chimpanzees from Central Africa" - Scientific Figure on ResearchGate. Available from: https://www.researchgate.net/figure/The-Amplification-strategy-and-the-genome-structure-of-the-new-SIVcpz-identified_fig7_230865090 [accessed 28 Apr, 2020]Figure B is adapted from 'A novel chimeric Rev, Tat, and Nef (Retanef) antigen as a component of an SIV/HIV vaccine," by Zdeněk Hel, Julie M. Johnson, Elzbieta Tryniszewska, Wen-Po Tsai, Robert Harrod, Jake Fullen, JimTartaglia, and Genoveffa Franchini, 2002, *Vaccine,* 20, 3174.

HIV Viral Replication

HIV targets CD4+ T cells and gains entry by attaching to the CD4+ receptor on the surface of these cells (Naif, 2013). This internalization occurs via the surface protein, gp120. This action triggers a series of conformational changes that assist in viral attachment to the host cell. The virus is then able to bind to a chemokine co-receptor, which triggers an irreversible conformational change that results in the formation of a pore by gp41. The subsequent fusion of the viral envelope to the plasma membrane releases the viral capsid into the cytoplasm of the host cell (Simon et al., 2006). In the cytoplasm, the virus uses its reverse transcriptase to transcribe its RNA genome into proviral DNA. Finally, the proviral DNA is transported to the nucleus and integrated into the host cell genome via an integrase., establishing latency. HIV viral reverse transcriptase has no proofreading activity, which causes additional mutations in the proviral DNA. These mutations lead to viral particles with variant genomes that are able to evade the immune system, resulting in a sustained infection (German Advisory Committee Blood, 2016).

HIV Illness & Symptoms

HIV is a chronic infection that causes a depletion of CD4+ lymphocytes, which leads to a weak immune system resulting in a variety of non-specific symptoms. During the initial symptomatic phase, which lasts 2-6 weeks, infected individuals will show non-specific symptoms similar to EBV or CMV induced mononucleosis. More serious symptoms can include aseptic meningitis, meningismus, and photophobia. Individuals may also present with early symptoms including lymphadenopathy, or oral

manifestations such as leukoplakia, thrush, or periodontal disease. Viral titers are highly infectious during this time. However, during the asymptomatic period, the virus enters a latent period, and the majority of patients will not show signs or symptoms until years after exposure to HIV. Even though viral titers are low during the asymptomatic period, individuals may still unknowingly spread the disease. Since there is a broad range of early signs and symptoms, physicians must be vigilant in assessing patient history and risk factors to recognize an HIV infection before it manifests into AIDS. (Miedzinski, 1992).

If left untreated, HIV can cause significant CD4+ cell depletion that can lead to AIDS within 10 years from initial infection (German Advisory Committee Blood, 2016). This weakened immune system leaves the body susceptible to opportunistic infections such as *Pneumocystis carinii* pneumonia (PCP), which is an indicating factor for AIDS. Additionally, patients can contract tuberculosis, which can be fatal in immunocompromised individuals. Neurologic effects include AIDS dementia complex, which occurs in 60% of AIDS cases. These diseases are the indirect result of an HIV infection, and many deaths occur due to the secondary opportunistic infections caused by a weakened immune system. Another sign that is indicative of AIDS is Kaposi's sarcoma, a malignant cancer that causes nodules on mucous membranes, or any organ (Miedzinski, 1992).

HIV Diagnosis

During the initial asymptomatic phase, infected individuals will not show any symptoms. Due to this reason, the early recognition ofpotential infections are made by

assessing the patient's risk factors such as intravenous drug use, sexual contact, or exposure to blood products. During this phase, a test for the p24 HIV core antigen can be conducted in the early stages if the assessment of risk factors forms enough evidence to suspect an infection (Miedzinski,1992). Primary screening for HIV occurs by testing for antibodies against the virus or detecting the virus. Antibody or antigen detection by using a serological test is more commonly used because it is rapid and can be done on various bodily fluids such as plasma, blood, or saliva. However, these tests are not effective when the virus is in the latent phase because no antibodies are present. Babies born to mothers who are infected will have maternal HIV antibodies, and a test may result in a false-positive. In these situations, the viral DNA in the host's genome has to be detected using RT-PCR (Simon et al., 2016). Current methods of HIV antibody are sensitive enough to detect the virus within 1-2 weeks of infection, which allows the individual early access to treatment and prevents further transmission of HIV (Zulfiqar et al., 2017).

In order to determine the severity of the infection and progression towards AIDS, physicians have to determine the stage of the disease by measuring CD4+ cell count and plasma viral load using flow cytometry. Other symptoms associated with opportunistic infections are also evaluated. The time between specimen collection and laboratory assays should be minimal, so dried blood samples are used to counteract problems associated with the transportation of specimens. However, communities, where resources are limited, need more cost-effective methods for analysis such as dipstick assays, total white-counts, or CD4+ chips (Simon et al., 2006).

HIV Treatment and Prevention

The current treatments for HIV do not eradicate the virus. Instead, the treatments help prevent the progression of HIV to AIDS and HIV infection to high-risk individuals. Initially, HIV was treated with only one antiretroviral drug. As the development and discovery of HIV drugs increased to include various inhibitors of HIV essential enzymes, more drugs started to be used in combination. This combination drug therapy became known as HAART (highly active antiretroviral therapy). The main goal of this therapy is to increase the strength of the immune system by reducing viral load and increasing CD4+ T lymphocytes (Zulfiqar et al., 2017). This treatment is effective in controlling viral multiplication and prolongs the asymptomatic stage of the infection resulting in lower risk of transmission and progression to AIDS (Bhatti et al., 2016). Because HIV manifests various symptoms in different individuals, the drug combinations are modified according to the patient. Normally, HAART involves a combination of three drugs, which can include reverse transcriptase inhibitors, protease inhibitors, fusion inhibitors, chemokine receptor 5 antagonists, or integrase transfer inhibitors. Each of these drugs targets viral entry, integration of viral DNA into the host's genome, or the production and assembly of the virus. HAART is recommended for all HIV patients regardless of the CD4+ lymphocyte count, and adherence to the regimen is recommended to prevent drug resistance and viral rebound (Bhatti et al., 2016).

HIV can be prevented by avoiding high-risk behaviors such as intravenous drug use, multiple sexual partners, and unprotected sex. For high-risk individuals, another option is pre-exposure prophylaxis (PrEP), which is used in uninfected individuals before potential exposure to HIV. This treatment prevents HIV infection by inhibiting viral

replication once the virus enters the body, and it prevents HIV from establishing a permanent infection. Truvada, which is a combination of two drugs, is usually the drug of choice for PrEP (Marfatia et al., 2017). When taken consistently, PrEP is an effective way to prevent new HIV infections (Eakle et al., 2018). Post-exposure prophylaxis (PEP) is used in emergencies after exposure to potentially HIV infected individuals or needles. PEP should be used within 2 to 72 hours of HIV exposure for maximum efficacy and continued for 28 days post-exposure. PrEP and PEP are useful treatments for the prevention of HIV infection when used correctly and consistently (Marfatia et al., 2017).

CHAPTER 2: PLASMID-BASED DNA VACCINES

Current vaccination strategies involve using vaccines containing live, attenuated viruses, killed pathogens, or viral subunits. Killed pathogens provide immunity through the humoral response by mobilizing CD4+ lymphocytes; however they do not provide permanent immunity. Live, attenuated vaccines provide both cellular and humoral immunity, and they provide life-long immunity. However, there are disadvantages to live, attenuated vaccines as well. A live, attenuated vaccine only targets a specific viral subtype, therefore it is not effective against a virus with multiple subtypes. Additionally, the degree of attenuation affects the strength of the immune response. There is also the risk of an attenuated vaccine recovering virulence and causing disease. Due to these limitations, scientists were driven to develop new vaccination strategies such as DNA vaccines (Ferraro et al., 2011).

Background

DNA vaccines became of interest in 1990, when it was discovered that plasmid DNA could induce immune responses to viral and nonviral antigens. Initial clinical trials included DNA vaccines against HIV, influenza, human papillomavirus, hepatitis, and malaria. However, these first generation DNA vaccines produced low CD4+ and CD8+ lymphocyte responses resulting in poor immunogenicity. This low immunogenicity was hypothesized to be from inefficient plasmid delivery methods. Following these results,

many improvements were made to optimize transfection efficiency, which led to the development of second-generation DNA vaccines (Ferraro et al., 2011).

In contrast to the conventional vaccines, DNA vaccines contain genes for a particular antigen rather than the antigen itself. This way, the protein produced by the gene sequence can be introduced into the MHC class 1 processing pathway for presentation to CD8+ lymphocytes. With DNA vaccines for HIV, the viral RNA is converted to DNA, which is then inserted into a bacterial plasmid. This plasmid is then introduced into a bacterial vector where it can be used as a vaccine and be administered intramuscularly. After administering the vaccine, the plasmid is taken up by the cell's nucleus, where the promoter will initiate transcription, which is followed by protein production. These proteins mobilize T cells and antibodies that will destroy the virus. Since these proteins are created inside the cell, they are also exposed to different antigen processing pathways that involve MHC class I molecules, thus expanding vaccines to include cellular responses as well as humoral responses (Donnelly et al., 2005).

DNA Vaccines for HIV

Developing a vaccine against HIV produces several unique challenges. The conserved receptor and co-receptor bindings sites that assist in viral attachment are covered in a glycosylated residue, which allows the virus to evade antibodies. Additionally, HIV is able to integrate into the host's genome and become latent, which allows the virus to escape the immune system. The high error rate of the reverse transcriptase has resulted in at least 12 viral subtypes, adding to the challenge of creating a universal vaccine (Gira et al., 2004).

Due to the high mutation rate and the complex nature of HIV, an effective vaccine for HIV requires cellular and humoral responses. Several vaccine trials have tested the heterologous prime-boost method, which combines a DNA-based and viral-based vaccine with a recombinant protein vaccine (Ferraro et al., 2011). The addition of a DNA vaccine induces a broader T-cell response, which is important in combating an infection that primarily infects helper T-cells (Donnelly et al., 2005). Currently, a trial determining the safety of an DNA vaccine containing HIV genes for *gag*, *pol*, and *env* is underway (Ferraro et al., 2011).

Specific Aims for this Experiment

This experiment involves using pVAX, a plasmid approved for use in developing DNA vaccines, with the gene sequence for SIV proteins. This gene sequence is collectively called Retanef, and is composed of genetically modified and re-assorted sequences for *rev*, *tat*, and *nef* proteins. These three proteins are expressed early in the viral life cycle, and their recognition may increase the elimination of infected cells by the immune system. The frequency of T-cell recognition of these three proteins was also shown to be higher than reverse transcriptase, env gp41, and gp120 proteins. The Rev protein contributes to viral replication, and the Tat protein down-regulates MHC class II proteins while promoting T-cell apoptosis. Tat also transactivates multiple genes that may contribute to the development of AIDS-related tumors. Nef is especially important to pathogenicity because it down-modulates MHC class I surface proteins, which contributes to viral evasiveness to the immune system by protecting virus-infected cells from apoptosis. Partially deleted Nef protein is able to re-arrange itself back into a

pathogenic form. By including the sequence for the Nef protein in this vaccine, recognition of the infected cells may occur before Nef down-modulates the MHC class 1 surface proteins and allow the immune system to clear the infected cells. The effect of these early regulatory genes on the host's MHC molecules indicates the importance for their inclusion in a vaccine that aims to increase immune T-cell response (Hel et al., 2002).

The structure of the Retanef coding sequence contains *rev*, *tat*, and *nef* SIV gene sequences. The *rev* and *tat* sequences are split into two segments to disrupt their functionality. The C-terminal part of the *tat* sequence is placed in between the C-terminal and N-terminal part of the *nef* sequence. The *nef* sequence was separated by the *tat* sequence to prevent the recombination of *nef* into its pathogenic form. An HA-tag was attached to C-terminal end of the entire sequence to facilitate the detection of the protein using antibodies. These details can be seen in Figure 1.

CHAPTER 3: METHODS

The methods in this thesis were used to qualitatively characterize a previously constructed pVAX1(Thermo-Fisher Scientific) vector (*Figure 2)* with SIV-retanef proteins using PCR (Polymerase Chain Reaction), immunofluorescence, and western blotting. The pVAX1 vector is specifically constructed for use in the development of a DNA vaccine. The plasmid vector is 3.0kb in size with a CMV(cytomegalovirus) promoter, which ensures a high level of expression in various mammalian cells. The plasmid vector has multiple unique cloning sites, however, the SIV-retanef gene has been inserted near the BamH1 restriction site, making it the point of interest in this study. SIV –retanef has been constructed by re-assortment of the *rev*, *tat*, and *nef* open-reading frames of SIV. There is also an HA (hemagglutinin) tag that has been inserted at the end of the SIV-retanef gene sequence for antibody detection during immunofluorescence or western blotting. There are two pVAX1-RTN (pVAX1-Retanef) clones that have been previously constructed with the SIV-retanef gene sequence in opposite orientations to the CMV promoter. The experiments conducted in this thesis analyze the orientation of the SIV-retanef gene in these two clones via PCR and successful expression of the protein by transfection into African green monkey kidney cells, also called Vero cells. The expression of the protein was analyzed by using immunofluorescence and western blotting.

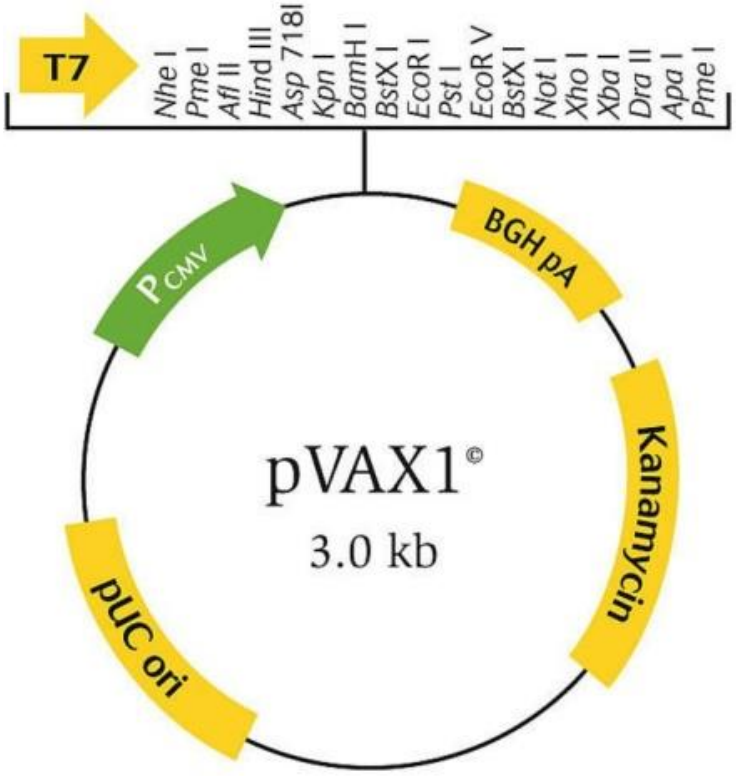


Figure 2- Structure of the PVAX1 vector – This figure shows the gene for Kanamycin resistsance, the location of the CMV promoter inside the pVAX1 vector., and the BamH1 restriction site. The size of the plasmid is 3.0 kb , and the size of the RTN protein is 1.486 kb or 55kDa.(https://www.thermofisher.com/order/catalog/product/V26020#/V26020)

Restriction Endonuclease Analysis of pVAX-RTN and PCR analysis of RTN

A restriction digest using BamHI, a restriction endonuclease, was used to isolate the gene sequence with SIV-retanef from the rest of the plasmid for pVAX-RTN clones 1 and 2. Five μL of pVAX-RTN clone 1 was added to an eppendorf tube along with two μL of CutSmart buffer, 1 μL of BamHI, and 12 μL of distilled water. This process was repeated for pVAX-RTN clone 2, and the two tubes were added to a water bath at 37℃ to cut overnight.

The following day, both pVAX-RTN clones were loaded onto a trisborate gel with a molecular weight ladder for gel electrophoresis. After completion, the gel was observed under an imager, which allowed visualization of results by showing the size of the cut pVAX vector and the SIV-retanef gene sequence in each of the two clones.

In order to determine the orientation of the SIV-retanef gene sequence, a PCR with forward and reverse primers for SIV-Retanef was conducted for both pVAX-RTN clones. Three PCR tubes were used with tube 1 containing, pVAX-RTN clone 1, CMV primer sequence, and a reverse RTN primer. Tube 2 contained pVAX-RTN clone 1, CMV primer sequence, and a forward RTN primer. Tube 3 contained pVAX-RTN clone 2, reverse RTN primer, and a forward RTN primer. The amount of DNA and primer sequences used in each tube was 2 μL. All three tubes contained 25 μL of the Bio Taq mix, and 19 μL of distilled water. A PCR was run on each tube, and this was repeated for pVAX-RTN clone 2.

Detection of SIV-Retanef Protein in Vero Cells via Immunofluorescence

Before starting the protocol for immunofluorescence, both pVAX-RTN clones were sent for gene sequencing to Eurofins, which verified the presence of a gene sequence of an HA tag for 1° antibody (Appendix A). The sequence also verified that the gene sequence inserted into the pVAX plasmid in both clones was indeed SIV-Retanef. Next, pVAX-RTN clones 1 and 2 were transfected into Vero cells using the Superfect reagent (Invitrogen) before observation under a Zeiss microscope using the immunofluorescence settings.

It is important to mention that before deciding to use a 4-well permanox chamber slide to transfect the Vero cells, a 96-well plate and 8-well chamber glass slide were also used. However, observation under a microscope using a 96-well plate did not produce conclusive results. The 8-well chamber glass slide resulted in the infected Vero cells peeling off the slide before antibody detection could occur. After trial and error, a 4-well permanox chamber slide produced successful adherence of infected Vero cells.

A 4-well permanox chamber slide was seeded to confluence with the outer two chambers having 40,000 and 60,000 cells each. The two middle chambers had 50,000 cells each. All chambers were filled with 500 μL of cell media and placed in a CO_2 incubator. The chamber with 40,000 cells was used as negative control for cell transfection, therefore it was not transfected with the either pVAX-RTN clone. Since pVAX-RTN clone 1 could not express the SIV-retanef protein because the gene sequence was oriented in the backwards direction, it was used as a negative control for protein expression and only added to one chamber with 50,000 cells. The last two chambers with 50,000 and 60,000 cells, respectively, were used for pVAX-RTN clone 2, which had the SIV-retanef gene sequence in the forwards direction.

The following methods explain the protocol for one chamber as per the manufacturer's directions for Superfect. Firstly, Optimem was used to dilute 1 μg of the plasmid DNA to a total volume of 60μL. The concentration of plasmid DNA for clone 1 and 2 was 0.5$\mu g/\mu L$ each, so a 2μL solution of each was used to obtain 1μg of plasmid DNA. Next, 5μL of Superfect was added to the plasmid DNA and optimum solution and mixed. This mixture was incubated at room temperature for 5-10 minutes. While the plasmid DNA-superfect complex formation was taking place, the growth medium was

aspirated from the 4-well permanox chamber slides. The cells in the chamber slide were washed once with 300μL of 5% PBS. After adding and mixing 350μL of cell media without trycine to the plasmid DNA-Superfect complexes, the total volume was transferred to the chamber slide. The cells with the transfection complexes were incubated for 3 hours at 37℃ in a CO_2 incubator. Afterward, the medium with remaining transfection complexes was removed, and the cells were washed once with 350μL PBS. Finally, 500μL of cell media was added to the cells before incubating at 37℃ in a CO_2 incubator for at least 48 hours.

After the incubation time was complete, the cells were fixed onto the slide in preparation for visualization using antibodies. After the cell media was removed from each chamber, 500 μL of 4% paraformaldehyde was added to each chamber and incubated for 20 minutes at room temperature. Next, the paraformaldehyde was removed, and the cells were permeabilized with 500 μL of 0.2% triton x-100 for 10 minutes at room temperature. The triton x-100 was removed before adding 500 μL per chamber of 1% BSA in PBS to block the cells overnight in the fridge at 4℃.

Next, the blocking buffer was removed from the cells, and the antibody dilutions were prepared. The 1° antibody used was an HA-tag monoclonal antibody designed to identify and adhere to the hemagglutinin tag on the expressed SIV-retanef protein. A 1:100 dilution was made with this antibody and 1% BSA in PBS before adding 100 μL to each chamber. The cells were then incubated for 2 hours at room temperature. After the 1° antibody dilutions were removed and the cells washed four times with 1X PBS, the 2° antibody dilutions were prepared using a goat anti-mouse antibody conjugated to FITC. All of the steps involving the 2° antibody were conducted in the dark due to the FITC

label's sensitivity to light. The 2° antibody was diluted in a 1:1000 ration using 1% BSA in PBS, and the cells were incubated with 100 μL of this dilution for 1 hour. After removing the 2° antibody and washing the cells again with 1X PBS, the cells' nuclei were stained with DAPI for 5-10 minutes. Lastly, the cells were washed again with 1X PBS after removing DAPI. The chamber walls were removed, and the slide was prepped with 70% glycerol and a coverslip for observation under the Zeiss scope for immunofluorescence.

Identification of SIV-Retanef Protein via Western Blotting

Vero cells were transfected with pVAX-RTN clones 1 and 2, and the protein expressed by the SIV-retanef gene was isolated and identified using western blotting. Vero cells were seeded in two 25cm^2 flasks. One flask was seeded with 16×10^5 cells, and transfected with pVAX-RTN clone 1. Another flask was seeded with 8×10^5 cells and transfected with pVAX-RTN clone 2. The same Superfect transfection protocol was used to transfect the cells with the measurements adjusted according to the size of the flasks.

After transfection, the cells in each flask were solubilized with 500μL of a 1:10 mixture DTT and protein solubilization buffer. The solubilized cells were transferred to separate tubes and placed in boiling water for 5 minutes. Next, the cells in both tubes were centrifuged for 5 minutes at 21,000xg in preparation for an SDS-PAGE, a common procedure used to separate proteins based on mass. The protein samples and molecular weight ladder were run on a 10% tris-glycine gel for 75 minutes at 100V and 60mA. The order that the proteins and molecular weight ladder were loaded is shown in Figure 3.

After separating the proteins on a gel, the samples were transferred to a PVDF membrane for antibody detection. Before starting the western blot, the blotting sponges and filter paper were soaked in 1X transfer buffer. The PVDF membrane was soaked in methanol for 30 seconds and washed with distilled water for 3-5 minutes. After the SDS-PAGE ended, the gel sandwich was assembled by putting the PVDF membrane on the gel. Afterward, the filter paper was added to the gel and the PVDF side of the sandwich. The blotting sponges were then placed on each side of the sandwich, covering the filter paper. This sandwich was then clamped inside a cassette, and the blotting transfer was run for 75 minutes at 20V and 180mA. After the transfer was complete, the PVDF membrane was washed with distilled water two times for 5 minutes each.

Before starting the chemiluminescence immunoassay, the PVDF membrane with the samples was washed with 100% methanol for a few seconds and washed with distilled water. Then, the membrane was blocked overnight in 5% blotto blocking buffer at 4℃. The next day, the 1° antibody was diluted 1:200 in 5% blotto blocking buffer. The primary antibody used was the HA-tag anti-mouse monoclonal antibody, and the membrane was incubated with this antibody at room temperature for 3 hours on a gyratory shaker. Afterward the 1° antibody was removed, and the membrane was washed with 1X PBS five times for 5 minutes each. Next, a 1:1000 dilution of 2° antibody and 5% blotto blocking buffer was placed on the membrane and incubated for 1 hour at room temperature on a gyratory shaker. The 2° antibody used was an HRP (horse radish peroxidase) conjugated goat-anti-mouse antibody. Next, the membrane was washed with 1X PBS five times for 5 minutes each. Next, a 1:1 solution of luminol and peroxide substrate was added to the membrane for 5 minutes to react with the horse radish

peroxidase enzyme, which emits light and is detectable by the Biorad Chemidoc Imager. The “high resolution” program was used to expose the membrane to light for five seconds, capturing an image every second.

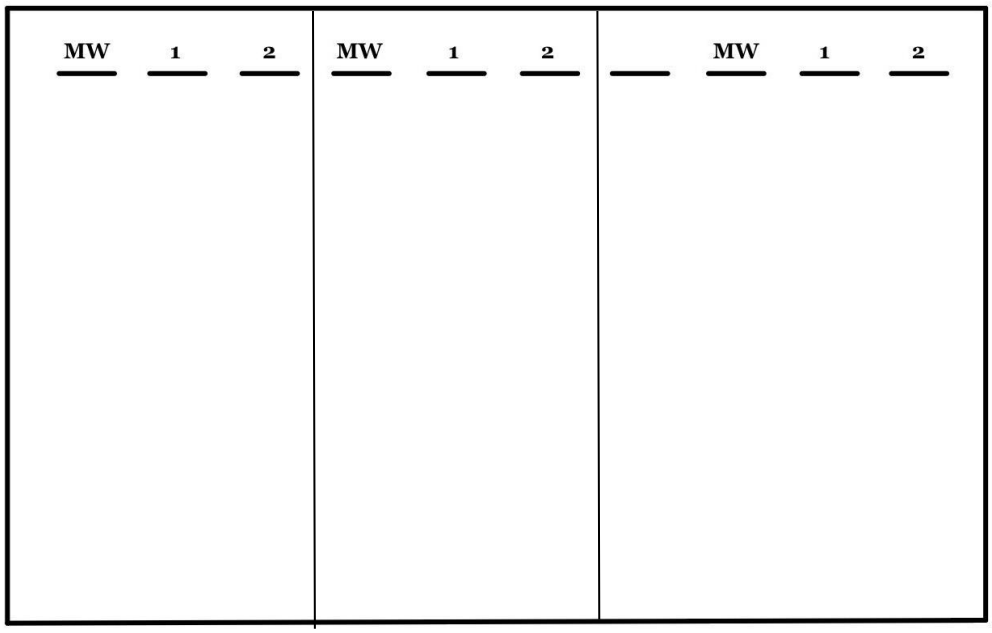


Figure 3 - Sequence of samples prior to performing SDS-PAGE. Molecular weight ladder was always loaded before loading pVAX-RTN clones 1 and 2. This PVDF membrane provided 3 blots, 2 of which were used in a later experiment with other antibodies.

Western Blotting using SIV positive monkey serum

The remaining blots from the previous western blot were used to do a chemiluminescence immunoassay using SIV positive serum from a monkey challenged with the macaque strain of SIV (SIVmac251) as the 1° antibody, and the 2 ° antibody was HRP-conjugated anti-monkey. SIV negative monkey serum was used as the 1° antibody, and the same 2 ° antibody was used. The same methods for a chemiluminescence immunoassay were followed, as mentioned in the Methods for western blotting.

RESULTS

Restriction Endonuclease Analysis of pVAX-RTN

The purpose of restriction endonuclease analysis of pVAX-RTN was to confirm the presence of the gene encoding Retanef in the BamH1 restriction site. After cutting the pVAX-RTN clones 1 and 2 with BamH1 restriction endonuclease and running the resulting samples on a trisborate gel, the results showed two bands. The 3.0kb band indicates the larger remaining sequence from the cut pVAX-RTN plasmid. The 1.5kb band indicates a shorter sequence within the BamH1 restriction site on the pVAX plasmid. These results shown in Figure 4 confirm that the Retanef gene sequence was inserted into the BamH1 restriction site on pVAX-RTN clones 1 and 2.

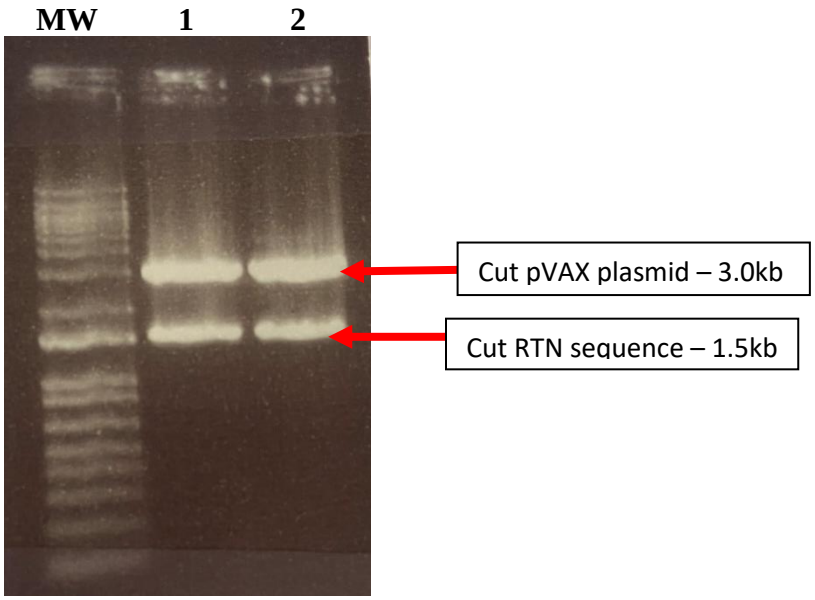


Figure 4 – Gel electrophoresis of pVAX-RTN clones 1 and 2 cut with BamHI. A molecular weight ladder was loaded before the pVAX-RTN clones. After cutting each clone with BamHI, the samples were loaded onto a trisborate gel for electrophoresis. Lane 1 contains pVAX-RTN clone 1, and lane 2 contains pVAX-RTN clone 2.

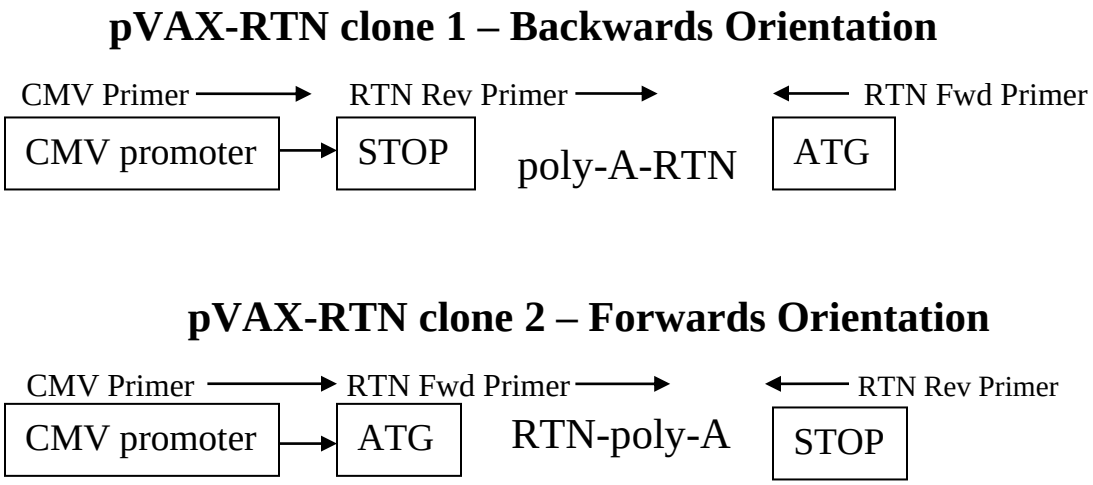


Figure 5 – PCR data using RTN forward and reverse primers. The figure above shows the orientation of the RTN forward and reverse primers for PCR analysis. The purpose of the PCR was to determine the orientation of the RTN gene sequence relative to the CMV promoter. The sequence in the backwards orientation was used as the negative control.

PCR analysis of SIV-RTN in pVAX-RTN

PCR was conducted to confirm that the 1.5kb gene sequence in pVAX-RTN clones 1 and 2 is the gene sequence for SIV-RTN. The use of RTN specific primers during PCR produced results that confirmed that the 1.5kb gene sequence inside clones 1 and 2 is SIV-Retanef. These results are shown in Lane 3 in Figures 6 and 7 where the forward and reverse RTN primers were used. The results in Lane 3 in Figures 6 and 7 confirm that the 1.5kb sequence is RTN in pVAX-RTN clones 1 and 2.

The RTN sequence inserted into the pVAX plasmid can be in the forwards or backwards orientation with respect to the CMV promoter. A PCR analysis with the specific combination of the primer for the CMV promoter, forward RTN primer, and reverse RTN primer was used to determine the orientation of the RTN sequence inside each clone. After running a PCR with specific RTN primers, and the specific pVAX-RTN clone, the resulting samples were run on a trisborate gel. Figure 6 shows the results for pVAX-RTN clone 1. Lanes 1 and 2 contained the experimental samples while lane 3 contained forward and reverse RTN primers. The results for the PCR sample in lane 1 with pVAX-RTN clone 1, CMV primer, and reverse RTN primer were inconclusive. Lane 1 does not have a definitive band throughout the gel. Lane 2 contains the PCR results for pVAX-RTN clone 1, CMV primer, and the forward primer; this lane shows a definitive band, which indicates that using a forward primer results in a successful amplification of pVAX-RTN clone 1. Lane 3 contains both forward and reverse primers for RTN, and the results show a clear band. The results shown in Figure 6 in Lane 2 and Lane 3 indicate that the RTN sequence in pVAX-RTN clone 1 is backwards relative to the CMV promoter, and the 1.5kb sequence in clone 1 is the sequence for Retanef,

respectively. Therefore, pVAX-RTN clone 1 was used as a negative control for further experiments involving the expression of the Retanef protein. The orientation of the CMV primer, forwards RTN primer, and backwards RTN primer can be seen in Figure 5. Prior to doing PCR, the pVAX-RTN clones 1 and 2 were sent to Eurofins for gene sequencing, and these results also confirmed that the 1.5kb sequence is the sequence for Retanef (Appendix A).

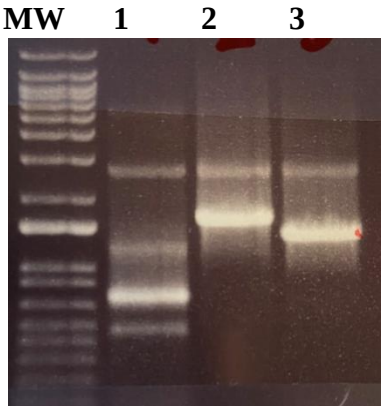


Figure 6 – Gel electrophoresis of pVAX-RTN clone 1 PCR products using forward and reverse primers. A molecular weight ladder was loaded before the pVAX-RTN clone 1 PCR products. Lane 1 contains the PCR products of pVAX-RTN clone 1, CMV primer, and the reverse RTN primer. Lane 2 contains the PCR products of pVAX-RTN clone 1, CMV primer, and forward RTN primer. Lane 3 is the positive control with the PCR products of pVAX-RTN clone 1, reverse RTN primer, and forward RTN primer.

The PCR results for pVAX-RTN clone 2 show conclusive results which can be seen in Figure 7. Lane 1 contains the PCR results for pVAX-RTN clone 1, reverse primer for RTN, and a CMV primer. This lane shows a clear band indicating that the reverse primer resulted in a successful amplification of pVAX-RTN clone 2. Lane 2 contains the forward primer, CMV primer, and pVAX-RTN clone 2. This lane does not have a clear band, which indicates the forward primer did not result in successful amplification of pVAX-RTN clone 2. Lane 3 is a positive control that contains both forward and reverse

primers, and the results show a clear band indicating successful amplification of pVAX-RTN clone 2. The results shown in Figure 7 in Lane 2 and Lane 3 indicate that the RTN sequence in pVAX-RTN clone 2 is forwards, and the 1.5kb sequence in clone 2 is the sequence for Retanef, respectively.

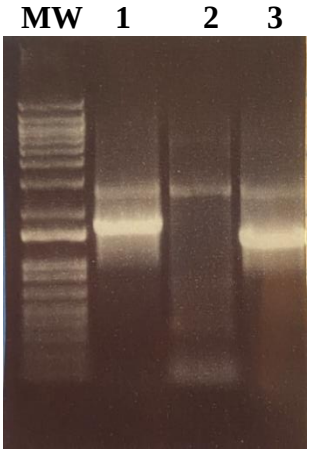


Figure 7 – Gel electrophoresis of pVAX-RTN clone 2 PCR products using forward and reverse primers. A molecular weight ladder was loaded before the pVAX-RTN clone 1 PCR products. Lane 1 contains the PCR products of pVAX-RTN clone 2, CMV primer, and the reverse RTN primer. Lane 2 contains the PCR products of pVAX-RTN clone 2, CMV primer, and forward RTN primer. Lane 3 is the positive control with the PCR products of pVAX-RTN clone 2, reverse RTN primer, and forward RTN primer.

Detection of SIV-Retanef Protein in Vero Cells via Immunofluorescence

After transfecting the pVAX-RTN clones into Vero cells, the expression of RTN protein inside Vero cells was observed using immunofluorescence. The transfected cells were stained with DAPI to analyze the location of the nuclei. Anti-HA-1 antibodies were the 1° antibody and used to localize the Retanef protein, and antibodies tagged with FITC were the 2° antibody and used to visualize the location of the 1° antibody. Vero cells transfected with pVAX-RTN clone 1and Vero cells transfected with pVAX-RTN clone 2 both showed viable cells when stained with DAPI. However, cells stained with FITC and

pVAX-RTN clone 1 did not fluoresce. The immunofluorescence results for Vero cells stained with FITC and transfected with pVAX-RTN clone 2 showed localization of the Retanef protein as indicated by the green fluorescence. These results shown in Figure 8 confirm that the transfection of pVAX-RTN clone 2 into Vero cells results in the successful expression of Retanef protein. The transfection of pVAX-RTN clone 1 into Vero cells does not produce Retanef protein. This was the expected result for pVAX-RTN clone 1 because it was used as the negative control.

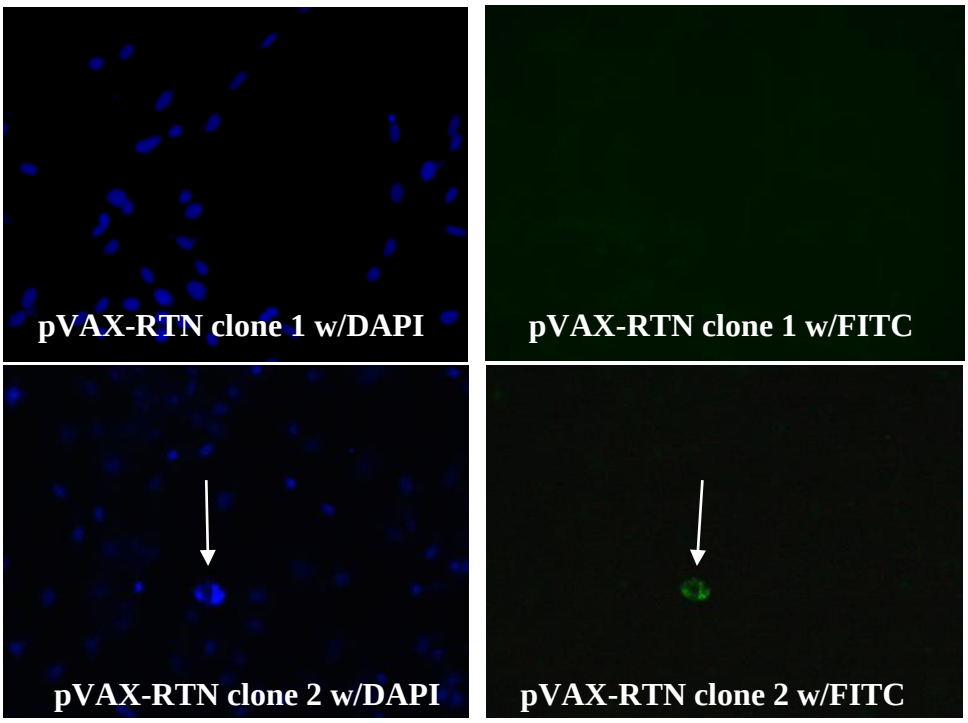


Figure 8 – Immunofluorescence analyses of Vero cells transfected with pVAX-RTN clones 1 and 2. The nuclei were stained with DAPI (blue fluorescence). The Retanef protein was stained with anti-HA-1 antibody and FITC-conjugated goat anti-mouse antibody (green fluorescence). Localization of the Retanef protein and stained nuclei of the cell is indicated with a white pointer arrow.

Identification of SIV-Retanef Protein via Western Blotting

Western Blotting was used to confirm further that the protein identified during immunofluorescence was Retanef. Vero cells transfected with pVAX-RTN clones 1 and 2 were solubilized, and the proteins were separated on a SDS-PAGE. After transferring the proteins to a PVDF membrane, the protein of interest was identified using antibodies in a chemiluminescence immunoassay. The 1° antibody used was a anti-HA-1 antibody, and the 2° antibody used was an HRP-conjugated antibody. Lane 1 was the negative control and contained proteins isolated from Vero cells transfected with pVAX-RTN clone 1, and the results did not indicate the presence of the Retanef protein. Lane 2 contained the experimental group which contained proteins isolated from Vero cells transfected with pVAX-RTN clone 2. This lane contained a definitive band around 55kDa, the estimated size of the Retanef protein. These results shown in Figure 9 indicate that the protein isolated from Vero cells transfected with pVAX-RTN clone 2 reacts to the anti-HA-1 antibody. Since the antibody can only identify the HA-tag on the Retanef protein and not the protein itself, this experiment can only definitively confirm the presence of the HA-tag. The next experiment further proves that the protein identified is Retanef.

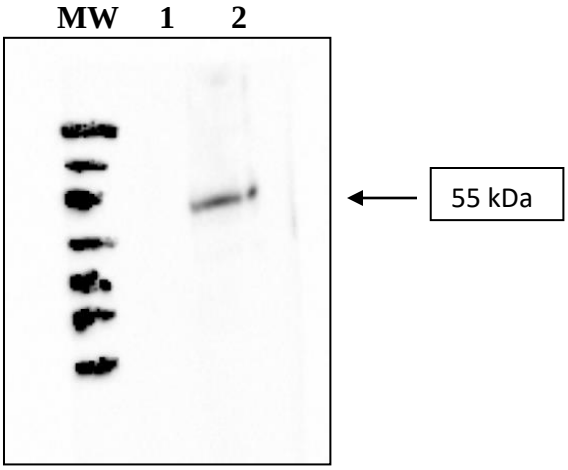


Figure 9 – Western Blot of pVAX-RTN clones 1 and 2 using anti-HA-1 antibody and HRP-conjugated anti-mouse antibody. Lane 1 has pVAX-RTN clone 1 as the negative control. Lane 2 contains pVAX-RTN clone 2 and is the experiemental group. There is a band around 55 kDa in lane 2; this is similar to the size of the Retanef protein.

Western Blotting using SIV monkey serum

Serum from monkeys infected with SIV was used to confirm the identity and analyze the humoral response produced by the protein isolated from the transfected Vero cells. SIV positive monkey serum was used against both clones, and the results are shown in Figure 10 (A). SIV negative monkey serum was also used against both clones as a negative control, and the results are shown in Figure 10 (B). Again, Vero cells transfected with pVAX-RTN clone 1 were used as the negative control for the expression of RTN protein, and the results for both SIV positive and SIV negative monkey serum showed no indication of the Retanef protein. Vero cells transfected with pVAX- RTN clone 2 reacted with SIV positive monkey serum, and the results are indicated by a 55kDa band found in lane 2 of Figure 10 (A). Vero cells transfected with pVAX-RTN clone 2 exposed to SIV negative monkey serum did not show any results and is indicated by the absence of a band in lane 2 in Figure 10 (B). The results for this experiment confirmed

that the protein produced by pVAX-RTN clone 2 is Retanef and that the protein is able to produce a humoral response with SIV antibodies.

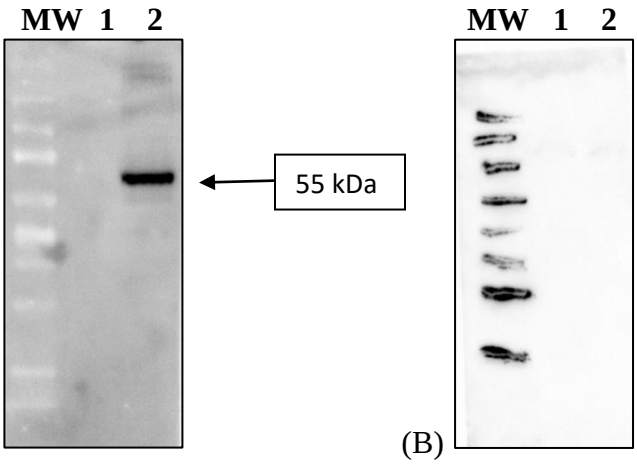


Figure 10 – Western Blot of pVAX-RTN clones 1 and 2 using SIV positive and SIV negative monkey sera. Both figures have pVAX-RTN clone 1 in lane 1 as the negative control, and pVAX-RTN clone 2 in lane 2 as the experimental group. (A) Monkey serum from a confirmed SIV positive monkey was used as the primary antibody to confirm if the protein isolated was an SIV protein. The protein identified had a band around 55kDa.(B) Monkey serum from a SIV negative monkey was used as the negative control.

CONCLUSION

Throughout this research process, different methods were used to characterize a plasmid-based DNA vaccine for SIV, and the results proved that the previously constructed pVAX-RTN plasmid successfully produces the Retanef protein inside Vero cells. The restriction digests and PCR resulted in successful identification of the identity and orientation of the Retanef gene sequence inside the pVAX plasmid. PCR results with pVAX-RTN clone 1 were only successful when the forwards RTN primer and CMV primer were used. For pVAX-RTN clone 2, the backwards RTN primer and CMV primer produced successful results. This validates that the pVAX-RTN clone 1 gene sequence for RTN is in the backward direction, and that the pVAX-RTN clone 2 gene sequence for RTN is in the forward direction. Following the conclusion made by PCR and restriction digests, pVAX-RTN clone 1 was used as the negative control because the results showed that the RTN gene sequence is backwards. This reverse gene sequence does not allow expression of the protein. The experimental group was pVAX-RTN clone 2, which is expected to successfully express Retanef protein due to its forward RTN gene sequence.

Localization and identification of the Retanef protein was successfully observed via immunofluorescence and western blotting using antibodies. However, the antibodies were only specific to the HA-1 tag on the Retanef protein, which does not prove that the Retanef protein is what was identified by immunofluorescence and western blotting. In order to prove that the protein identified was Retanef, serum from SIV positive monkeys was used in a western blot, and the results indicated that there was a reaction between

SIV positive serum and the isolated Retanef protein. This further proves that the protein expressed inside the transfected Vero cells is indeed Retanef.

The SIV positive serum produces a general reaction towards any SIV antigen; this experiment did not indicate if the protein expressed was *rev*, *tat*, or *nef*. Further studies can be conducted to isolate and determine the identity of the protein expressed. The results for the SIV positive serum also conclude that the protein expressed by the pVAX-RTN plasmid is successful in reacting to an immune response against SIV. Additionally, the cellular and humoral immune responses can be analyzed in further studies by challenging non-human primates with the pVAX-RTN plasmid. The pVAX-RTN plasmid proves to be a good candidate for expressing Retanef protein inside Vero cells, and further studies should be done to observe T-cell response. The results of this study show that the pVAX-RTN plasmid is a good candidate for a DNA vaccine against SIV, however further studies need to be conducted to observe the safety and efficacy of this vaccine as a therapeutic and prophylactic treatment for SIV-infected non-human primates.

Since most vaccine research against HIV has focused on using structural genes, the results of this experiment may encourage further research on including regulatory proteins in future vaccines against HIV. The data collected in this experiment provides strong evidence that a corresponding HIV vaccine with the *rev*, *tat*, and *nef* regulatory proteins should be evaluated. The overall goal of this experiment was to contribute towards HIV research and finding preventative treatments and vaccines for HIV by using SIV as a model.

APPENDIX

>PVAX_RTN1_CMV_CMVf Sample_Name=PVAX_RTN1_CMV_CMVf Chromat_id=10441183 Read_id=10350013 Version=1 Length=956

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CAGGCCCTTCTTCAGGAAGCAGAACTGGCAGTGGTAGCAGCACTTCTTGCAGTAGCAGGTGTTGTAGCA
GGCCTCCAGGGGGCGGTACAGCTGGCTCAGGATCTCCTCGCCCAGGTTGGCGCTCTCGGGGGTGGTGG
CGTCGGCCTCCAGGATGCAGCTGCTGCGCTCGTTGCTGCTCTCCAGGCTGTTCTCCTGCTCGCGCAGGGG
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AGCAGGCGGCCGTAGGTCTCGCCCCGGGCGCGCAGCAGGCGCTGGCGCAGGTCGCCGCTGGGGCGGCT
GCGGCGCATGCTGATGGCGTCGACGCCGCCACTAGTGCGGCCCAGGCCCGGGGGCGGTGGCCCCGGCC
TTCTCCCCGGTCTCCTTCTTGGCCTTCTCGGGCTGGNANNGNCGGGNGCGNTGGGG

Figure A-1- Gene sequencing results for pVAX-RTN clone 1. This figure shows the direction of the TGA stop site in relation to the CMV promoter in pVAX-RTN clone 1. Since this is sequence is in the reverse direction to the CMV promoter, the sequencing results are the reverse complement of the Retanef sequence. The highlighted portion is the reverse complement of the TGA stop site.

>PVAX_RTN2_CMV_CMVf Sample_Name=PVAX_RTN2_CMV_CMVf Chromat_id=10441185 Read_id=10350035 Version=1 Length=925

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GCCACTGCCAGCCCGAGAAGGCCAAGAAGGAGACCGTGGAGAAGGCCNNGGGCCACCGCCCCCGGCN
NGGGGCGCCCNNNNGGCGGNNNCNANNCNTT

Figure A-2- Gene sequencing results for pVAX-RTN clone 2. This figure shows the direction of the ATG start site in relation to the CMV promoter in pVAX-RTN clone 2. The ATG start site is in forwards orientation to the CMV promoter.

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