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Generating Zika Vaccine Candidates Using Nhumirim Virus as a Backbone

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Generating Zika Vaccine Candidates Using Nhumirim Virus as a Backbone

by

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Abstract

Zika virus (ZIKV) is a mosquito-transmitted flavivirus that is causing significant world-wide health concern. There are currently no treatments or vaccines available for this virus, thus, there is an urgent need to develop a safe and effective vaccine to combat ZIKV infection. Nhumirim virus (NHUV) is also a mosquito-transmitted flavivirus, but it is unable to infect humans and other vertebrate animals, making it an ideal candidate to develop chimeric viral vaccines against other disease-causing flaviviruses, such as ZIKV. In this study, we generated chimeric viruses by replacing *envelope (E)* gene in the genome of NHUV with *ZIKV E* gene, which encodes the major viral surface glycoproteins that mediate cellular receptor binding and induce host protective immune responses. The recombinant NHUV genomes were transfected into mosquito C6/36 cells in the forms of plasmids. The transfected cell supernatants were then collected and used to inoculate a new batch of C6/36 cells on day 4 post-transfection. The cell supernatants were continuously passed for five passages on C6/36 cells. Although a cytopathic effect (CPE) was not observed on the days of collection, the presence of the chimeric viral genomes of the chimeric viruses was confirmed by using RT-qPCR measuring ZIKV *E* and *nonstructural gene 1 (NS1)* of NHUV. The results of this study indicate the successful generation of chimeric ZNHUV viruses that can be further evaluated in cell culture and in a mouse model as a potentially safe and effective vaccine candidate against ZIKV infection.

Keywords: Vaccine, Zika, Nhumirim, Insect-Specific Flavivirus, Transfection, Virus

Dedication

First of all, I would like to dedicate this to my mother, Olatinuke Atobiloye, without whom I will not be where I am today. She has constantly supported me in every way possible and I could not be more grateful. To the rest of my family and friends who have contributed to the smooth sailing of my undergraduate college career in one way or the other, I dedicate this to you too. Thank you all so much. Finally, I would like to dedicate this to every young black girl who is trying to find her way in the STEM world. You are not alone, and you can be your own motivator.

Acknowledgments

I would like to thank Dr. Fengwei Bai, for being more than a thesis advisor to me. He has patiently guided me through this process, provided me with so many opportunities throughout my undergraduate years, and has taught me lessons in and out of the laboratory, and for that, I am forever grateful. Thanks to Biswas Neupane for his constant effort in teaching me the majority of the lab techniques I know. I would also like to acknowledge my other laboratory members, Farzana Nazneen, Christina Craig and Elizabeth Thompson for their support. Finally, special thanks to the Honors College and the School of BEES for their help with financial and moral support whenever I needed it throughout my research process. I am very grateful to you all.

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List of Abbreviations

CPE - Cytopathic Effect

DENV - Dengue Virus

EMEM - Eagle's Minimal Essential Medium

FBS - Fetal Bovine Serum

ISF – Insect-Specific Flavivirus

JEV - Japanese Encephalitis Virus

MBFV - Mosquito-Borne Flaviviruses

NHUV - Nhumirim Virus

PBS - Phosphate-Buffered Saline

P/S - Penicillin-Streptomycin

RNA - Ribonucleic Acid

RT-qPCR - Reverse Transcription Quantitative Polymerase Chain Reaction

WNV - West Nile Virus

ZIKV - Zika Virus

ZNHUV - Recombinant Zika and Nhumirim Virus

Chapter 1: Introduction

Zika Virus (ZIKV) belongs to the family Flaviviridae and has posed threats to the health of individuals, especially those in the Americas (9). Outbreaks of the other viruses that belong to the same family as ZIKV have also caused severe loss of lives and created a health and financial burden in other regions (9).

Insect-Specific Flaviviruses (ISFs) are a group of viruses from the Flaviviridae family that cannot infect humans and other mammals (9). These viruses replicate in mosquitoes; however, they will not infect vertebrates like humans (17). Cell Fusing Agent Virus (CFAV) was the first ISF to be discovered, and it has been used as a model to understand other ISFs, such as Long Pine Key Virus (LPKV) and Nhumirim Virus (NHUV) (9). As technological advances have increased over the years, additional ISFs are being discovered. These ISFs have been looked at as a means of combatting the flaviviruses damaging human populations.

In this project, a recombinant virus, in the form of a plasmid, was used to infect C6/36 mosquito cells that were derived from the mosquito species, *Aedes albopictus*. The envelope *E* gene of NHUV was replaced with that of ZIKV. We also examined the effects of the NHUV and inferred how this methodology could be applied to future studies in vaccine creation.

Chapter 2: Literature Review

Arboviruses are a group of viruses that use arthropods like mosquitoes as their vectors (23). In fact, research has shown that mosquitoes cause over ninety percent of arboviral related diseases in humans (9). Both positive-sensed and negative-sensed RNA viruses are found in this group, such as Flaviviridae and Bunyaviridae. The name "Flaviviridae" is obtained from the Latin word "flavus", which means "yellow" (4). Yellow fever virus is one of the most well-known viruses in this family. Virus genera in this family include *Flavivirus, Pestivirus* and *Hepacivirus* (4). There are more than 70 viruses in the genus of *Flavivirus,* and most of them are important human pathogens.

In the *Flavivirus* genus, the viruses are classified based on host/vector (9). The first group consists of insect-specific flaviviruses (ISF), which indicates that these viruses only infect insects. Since they are unlikely to grow in vertebrates, they use the process of vertical transmission to maintain their existence (17). Examples of ISFs include CFAV, LPKV, and NHUV (9). The other group of the *Flavivirus* genus consists of viruses that can infect both invertebrate insects and vertebrates. They are called dual-host Mosquito-Borne Flaviviruses (MBFVs) (17). Examples of MBFVs include Dengue Virus (DENV), Zika Virus (ZIKV), West Nile Virus (WNV) and Japanese Encephalitis Virus (JEV). Sometimes, flaviviruses are classified into a third cluster that has no known vector (NKV) (17). Apart from the host classification, flaviviruses can also be classified based on their epidemiology (9). Flaviviruses can cause a range of human diseases, such as Dengue Fever and Yellow Fever (9). The diseases and infections they cause range from neurologic diseases, to hemorrhagic diseases, to even mild febrile diseases (9). For example, ZIKV causes microcephaly in the unborn children of infected mothers and JEV

causes brain diseases. Geographic distribution, antigenicity, and nucleotide sequences are other means of classifying flaviviruses (10). Also, flaviviruses are diverse in terms of distribution, vectors, and hosts, which makes it more complex for them to be classified (19). In addition, we do not fully understand the broader ecological cycle of flaviviruses (4). Due to the advancement of molecular techniques, more flaviviruses are being discovered worldwide (20), thus the true number of flaviviruses that exist today is presently unknown.

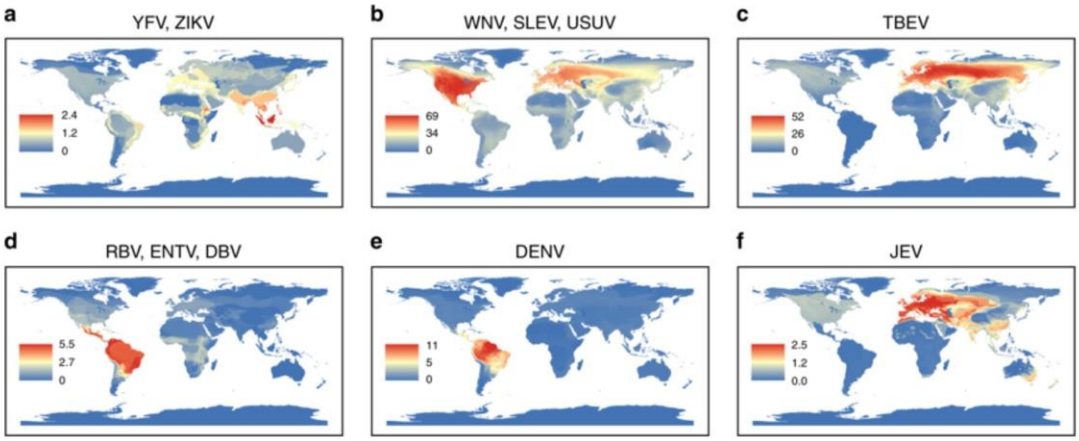


Figure 1: Geographical distribution of flaviviral host cell richness https://www.ucdavis.edu/news/where-will-worlds-next-zika-west-nile-or-dengue-virus-come/

Over 50% of the viruses in the flavivirus group are human pathogens that cause deadly diseases (4), and there is an urgent need to address and mitigate their threat to the human population. According to the World Health Organization, there are over 50 million new cases of Dengue fever per year. Likewise, deaths in Asia due to JEV and the Tick-Borne Encephalitis Virus in central Europe represent additional threats to people within these regions (4). The global distribution of the West Nile Virus (WNV) is also a

major threat, as it affects the human populations of Africa, Southern Europe, and North and South America (4). The lack of vaccines, drugs and methods of appropriate vector control is leading to the continued reemergence of these viruses (21).

As the years have gone by, additional flaviviruses have emerged and reemerged and their incidence level has increased (4). In 1952, Zika Virus (ZIKV) infection was first reported in humans (17). Before then, the virus had only been isolated in a Ugandan rhesus monkey in the Zika forest in 1947 (10, 7). Although ZIKV was initially isolated from the *Aedes africanus* mosquito, it was unclear whether it could infect humans until the first ZIKV related human illness was recognized in a Nigerian citizen (16). ZIKV is primarily transmitted via mosquitoes but it also can be passed on by other routes, such as sexual contact, mother-to-fetus transmission, and blood transfusions (Figure 2). ZIKV has been isolated from several species of mosquitoes including *Ae. africanus, Ae. aegypti*, *Ae. hensilli, Ae. furcifer,* and *Ae. apicoargenteus* (11). Similar to other flavivirus group members, ZIKV is also an RNA virus. It has a striking similarity to the Spondweni virus, having 10,794 nucleotides and 3,419 amino acids that it encodes (11). In terms of underlying pathogenesis, ZIKV is thought to replicate in dendritic cells, lymph nodes, and bloodstream, just like other mosquito-borne flaviviruses (11). Similar to many other flavivirus types, the incubation period of ZIKV is estimated to be less than one week (16). However, ZIKV replication during infection is different from other flaviviral types, as its antigens can be found in cell nuclei, rather than simply in the infected cell cytoplasm (11).

Zika Virus's African strain causes less severe illnesses compared to the Asian lineage strain, which is responsible for many severe symptoms, such as congenital

syndrome (17). Studies conducted in 1968 showed that 40% of the humans in Nigeria from whom ZIKV was isolated had neutralizing antibodies to the virus (11). In 2007, there was an outbreak of ZIKV that caused diseases with mild symptoms, such as conjunctivitis and arthralgia in a region of the Pacific Ocean called the Yap Islands (12), and more severe illnesses such as the Guillain-Barre syndrome were recorded (16). It was noted that the *Aedes hensilli* was the ZIKV vector responsible in the Yap Islands (16). This outbreak marked the first documentation of ZIKV detection outside of Africa and Asia (9, 13, 8), which was surprising as there were about 5000 infections documented (15, 8) from this outbreak. From the Yap Islands, several other outbreaks started to occur on other islands in the Pacific Ocean, such as the Easter and Cook Islands (15, 22, 18). Although there were significant cases documented before 2007, the Zika epidemic turned into a Zika pandemic between 2015 and 2016 (17).

Recently, ZIKV reemerged in some tropical regions, such as Brazil (17), and it has been noted that it was introduced from the Pacific Islands (16). The reemergence of ZIKV caused a global alarm because its infection could be linked to human birth defects, and this was only one of the major infectious diseases capable of causing such defect in more than half a century (16). Microcephaly, which is a condition associated with abnormal brain development and small head size (16), is one of the effects when ZIKV is passed from pregnant mothers to their fetus. The Brazilian Ministry of Health, the Centers for Disease Control and Prevention (CDC), and the World Health Organization issued an alert on the ZIKV-associated congenital malformation during the outbreak (17). The presence of ZIKV RNA has been confirmed both in the amniotic fluid and brain tissue of fetuses that developed microcephaly, hence there is a strong link between the

microcephaly deformity and Zika infection (16). Between September 2015 and February 2016, the cases of ZIKV-associated microcephaly in Brazil reportedly reached 4,300 (16). There have also been reported cases of peripartum related transmissions of ZIKV (16). In these cases, the symptoms range from some individuals being asymptomatic to others having rashes and illnesses (16). Sexual transmission has also been documented as a means of this viral spread (Figure 2). ZIKV viral particles have also been found in breast milk; however, transmission via breast milk has yet to be confirmed (16). It has been estimated that close to half of the world's population is at risk of becoming infected (9), therefore there is an urgent need to develop vaccines and antivirals.

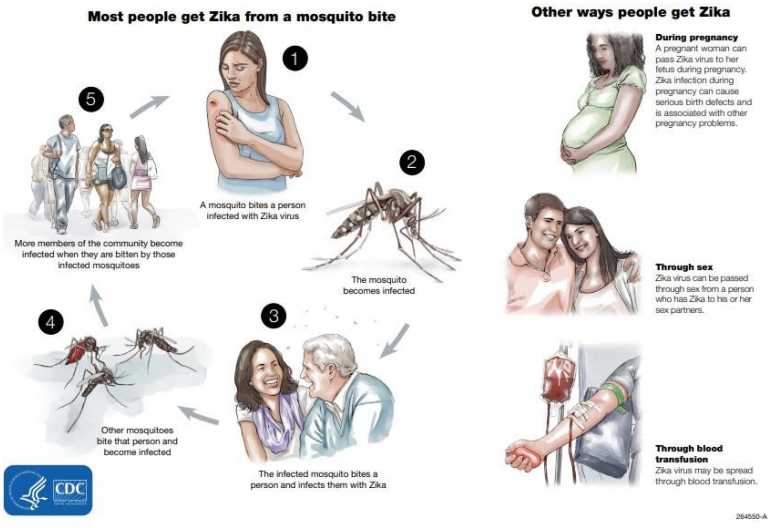


Figure 2: Transmission of Zika Virus (ZIKV) https://www.cdc.gov/zika/pdfs/Zika-Transmission-Infographic.pdf

As Insect-Specific Flaviviruses (ISFs), are unable to infect humans, they have been looked as a possible means by which vaccine development can be achieved. CFAV is the first flavivirus that was isolated from a line of *Aedes aegypti* cells (6). Thirty-eight ISFs have been documented as of November 2016, including NHUV and CFA, and many others are currently being discovered due to the advancement of molecular techniques (6).

NHUV was discovered in 2010 in *Culex chidesteri* mosquitoes (9) from the Brazilian Pantanal region (17). Previous studies suggest that NHUV may have lost its ability to replicate in vertebrate cells (9). In addition, some studies have shown that NHUV suppresses the growth of WNV (17). Furthermore, research has also shown that when NHUV is superinfected or co-inoculated with ZIKV, the growth of ZIKV is suppressed (17). NHUV can infect and replicate in C6/36 cells that were derived from *Aedes albopictus*, causing a cytopathic effect (CPE) (21). Plaque assays in C6/36 cells can be employed to determine NHUV titers in a viral stock (21). Likewise, C6/36 is suitable for studying other ISFs, and Vero cells derived from African green monkey kidneys are also a potential cell line used to study human and vertebrate viruses. Vero cells have been shown to lack the type I interferon response, making them susceptible to infection by many mammalian viruses (5).

Prior research has shown that when grown in cell culture, pathogenic flaviviruses may cause cytopathic effects; thus, if a vaccine is made to protect against a flavivirus, it needs to be safe (4). Other requirements for a good vaccine include thermal stability, ease of production, and low production cost (4). For some vaccines, prophylactic relevance will also be needed in order to combat more than one virus serotype (4). Thus, the development of a vaccine for DENV is particularly difficult because of its existence in multiple serotypes (4).

The genome of a flavivirus is approximately 11 kb in length, with an open reading frame that encodes three structural proteins and seven nonstructural proteins (Figure 3). Collectively, the various proteins of flaviviruses have individual roles in the viral replication process (3). When a flavivirus successfully infects a host, it translates its

genome into a polyprotein within the cytoplasm and is then subsequently cleaved by proteases to yield three structural proteins and seven nonstructural proteins (15). The C, prM, and E are the three structural proteins and NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 are the seven nonstructural proteins (9). The *NS1* gene was first reported as a viral antigen in Dengue patients in 1970 (14) and it is relatively conserved among all flaviviruses (14). NS1 is described as enigmatic since it has several oligomeric forms and also serves as a biomarker for detecting diseases early (14). NS1 is not restricted to one location in the cell (14). When it is intracellular, it plays a significant role in the viral replication process, but its specific function is currently unknown (14). In some studies, the NS1 glycosylation sites have been eliminated to create a hypothetic vaccine for some flaviviruses (1). The E protein has significant functions in helping the virus with host cell entry and assembly (15). For example, ZIKV in its mature form has been shown to contain 90 antiparallel E dimers when viewed in high resolution (15).

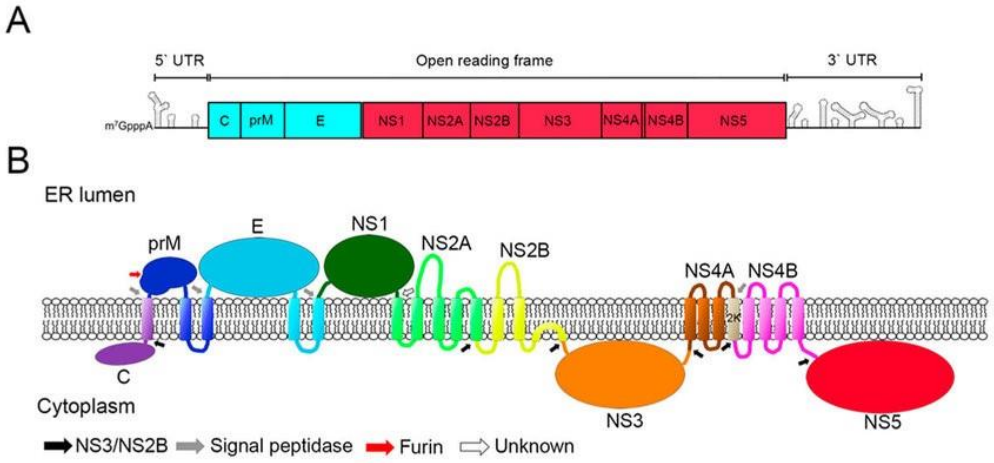


Figure 3: Flaviviral genome (2). A shows the single-stranded positive-sense RNA of a flavivirus with its seven nonstructural genes in red and three structural genes in blue. B shows the predicted transmembrane domains for the structural and nonstructural proteins.

Currently, the World Health Organization has reported approximately 38 ZIKV vaccine candidates and few of those have progressed to the clinical phase trial (12). It is

expected that a vaccine developed for ZIKV should have the capacity to not only prevent infection but to also protect the host against the pathological conditions that come with the viral infection (12). The types of vaccines developed include DNA and mRNA vaccines, attenuated vaccines, and viral-like particles or proteins (12). Each of these forms has its own advantages. For example, the live attenuated form has shown significant success in protecting against flaviviruses such as JEV, therefore it is expected that similar success will be observed with a ZIKV vaccine (12). In addition, the live attenuated forms are cost-effective and can induce the humoral and cellular immune responses within their hosts (12). The virus-like particles can induce antibody responses and are much stronger while the DNA vaccines are relatively easier to engineer and manufacture.

In this study, we utilized the NHUV genome as a backbone to replace the NHUV *E* gene with the ZIKV *E* gene to create a chimeric virus named ZNHUV. A plasmid was used in transfecting this ZNHUV into the mosquito cells. The envelopes of flaviviruses originate from the host cell lipid membrane during viral replication (3). The *E* gene specifically encodes for envelope proteins, which are glycoproteins that are inserted into the viral envelopes. These E proteins mediate cellular receptor binding and induce host protective immune responses. In this project, we transfected the plasmids of the chimeric ZNHUV genome into C6/36 mosquito cells and monitored the cytopathic effect. Subsequently, we used the RT-qPCR method to measure the presence of the ZIKV *E* gene and the nonstructural gene 1 (*NS1*) of NHUV in the cDNA. The results of this study will be used to determine if this chimeric virus methodology can be successfully used for ZIKV vaccine development.

Chapter 3: Methods and Materials

3.1. Experimental Outline Overview. The purpose of this study was to generate chimeric viruses by replacing the envelope *E* gene in the NHUV genome with the *ZIKV E* gene. After transfection, the peak day of infection for NHUV was first determined by RT-qPCR and plaque assay. The time with the highest viral genome concentrations was then used to collect the recombinant viruses. The transfected cells and supernatants were collected and used to inoculate a new batch of C6/36 cells for five generations. Following the collection of the cells from each passage, RT-qPCR was used to measure the presence of both the *ZIKV E* and the nonstructural gene 1 (*NS1*) of NHUV.

3.2. Cell Subculture. C6/36 cells were obtained from ATCC (CRL-1660™) and cultured in the growth medium, EMEM, with 10% FBS and 1% P/S in an incubator at 28°C with 5% CO_2. For subculture, the EMEM medium from the growth flask was discarded and the cells were washed with PBS. The PBS used for washing was again discarded and another 9 ml was added together with 1 ml of Trypsin-EDTA. The flask was then placed in the incubator for 5 minutes until the cells had successfully visibly detached from the flask's surface. Next, 10 ml of EMEM with 10% FBS and 1% P/S was added, and the mixture was transferred into a 50 ml centrifuge tube. The mixture was centrifuged for 5 minutes at 1500 rpm and after centrifugation, the supernatant was discarded through decantation. The resulting cell pellet was diluted with 10 ml of EMEM and then a subsample was re-added into the flask for further incubation. The remaining sample was counted and used for plating purposes.

3.3. C6/36 Viral Infection with NHUV. Healthy C6/36 cells were plated in 6-well plates following 3 days of growth. Approximately 1×10^6 cells were added per well and incubated overnight to achieve a monolayer of 100% confluency. The growth medium was removed from the confluent C6/36 cells after one day and replaced with 1ml of the viral growth medium. The cells were incubated with NHUV for an additional hour at 28°C. After incubation, 1 ml of fresh cell growth medium was added, and the cells were allowed to continue incubation. Between days 4 and 5 when cytopathic effects were observed, the supernatant was collected and used to further infect the next batch of cells.

3.4. Plaque Assay of NHUV. Plaque assay was used to calculate the plaque-forming units of NHUV, essentially the viral titer. To do this, the above viral infection process was followed. After one hour of the incubation, the supernatant was removed and a mixture of plaque agarose and EMEM medium was applied on the cells. After solidification at room temperature, the plates were incubated for 3~4 days. Once plaques started to form, the cells were stained with neutral red. The plaques were counted under bright light and the plaque-forming units (pfus) were calculated.

3.5. Transfection of C6/36 cells with ZNHUV. Healthy C6/36 cells were plated at an amount of 1×10^6 per well in a 6-well plate and incubated in a 28°C incubator with 5% CO_2 overnight. The following day, 5 µl of Flyfectin reagent (OZ Biosciences) was mixed with 125 µl of Opti-MEM medium in a sterile Eppendorf tube by pipetting up and down several of times. In another Eppendorf tube, 2 µl of plasmid DNA of ZNHUV was diluted in 125 µl of Opti-MEM medium. Two µl were added and mixed well again by pipetting

up and down. After, the diluted DNA was added into the tube containing diluted Flyfectin and the solutions were mixed well one more time. This mixture was incubated for 15 minutes at room temperature in a biosafety hood.

The incubated C6/36 cells from the previous day were gently washed with antibiotic-free EMEM-FBS medium, then 1ml of the EMEM medium was added per well and left to sit. After the 15-minute incubation period, the mixture of Flyfectin and ZNHUV plasmid was added to the individual wells containing C6/36 cells and incubated at 37°C for 8 hours. After incubation, the antibiotics free media in each well was removed and replaced with 2ml of growth medium. The cells were then kept in a 28°C incubator for 7 days after which, the supernatant, which contained viral particles, was collected to be used for secondary infection assays.

3.6. Supernatant Collection and RNA Isolation. After proper labeling of the tubes, the medium supernatant from each well was collected into individual Eppendorf tubes and centrifuged at 1500 rpm for 10 minutes at 4°C. Next, the supernatant was carefully transferred into a clean sterile Eppendorf tube. To extract total RNA from the cells, an RNeasy extraction kit (Qiagen) was used following the manufacturer protocols. The total RNA was then reverse transcribed into first strand cDNA using the iScript cDNA synthesis kit (BioRad).

3.7. Quantitative RT-qPCR. A real-time qPCR amplification was performed to amplify both the NHUV *NS1* and the ZIKV *E* genes. A probe-based qPCR was carried out for the ZIKV *E* gene investigation, while a SYBR green-based PCR was carried out for the

NHUV *NS1* gene. The standard curves were acquired using serial dilutions of a standard with known concentration. Mosquito housekeeping gene *Rp7* was used as an internal control for NHUV. For the qPCR conditions, the first step involved the samples running at 95°C for 5 minutes, then for 25 seconds at 95°C and 60°C for 1 minute in the second step, and for the final step, the second step was repeated for 40 cycles. The qPCR primers and probes were synthesized by Integrated DNA Technologies (IDT). All the qPCR reagents were purchased from Bio-Rad.

3.8. Statistical Analysis. The data collected were analyzed using analysis of variance (ANOVA) in GraphPad Prism 6 software. The ANOVA analysis was followed by Tukey's test.

Chapter 4: Results

4.1. NHUV Causes Significant CPE in C6/36 cells

C6/36 mosquito cells were subcultured with the EMEM cell growth medium. After plating in six-well plates, the cells were left to grow overnight in EMEM, 10% FBS and 1% Penicillin-Streptomycin until a monolayer formed with almost 100% confluency. The cells were then infected with NHUV and incubated for a week. The uninfected plate that was used as a control showed continuous growth of small, rounded C6/36 cells (Figure 4), while those infected with NHUV showed a significant cytopathic effect (CPE) (Figure 4) as the cells clumped and fused together. As shown in Figure 5, the higher the viral concentration, the greater the CPE seen (Table 1). Plaque assays were performed, and they showed that the titer of the NHUV stock was 6.5×10^6 pfu/ml using the formula:

$$\text{pfu} = \frac{Average\ plaques\ formed}{4} \times coefficient\ of\ dilution\ factor$$

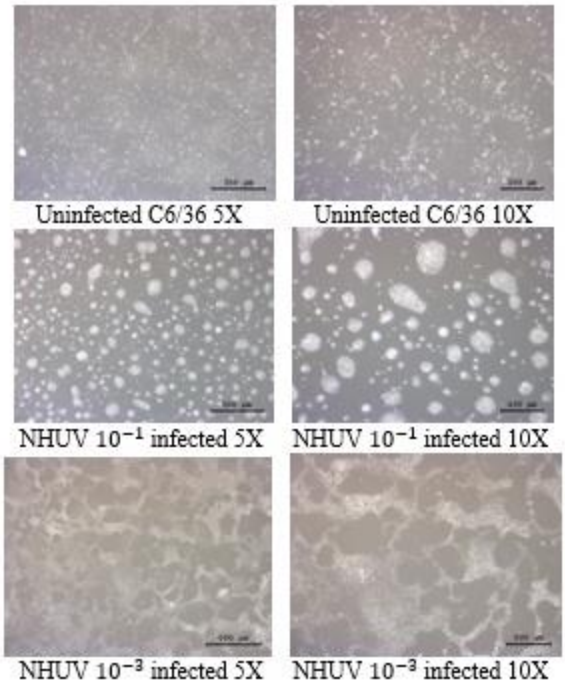
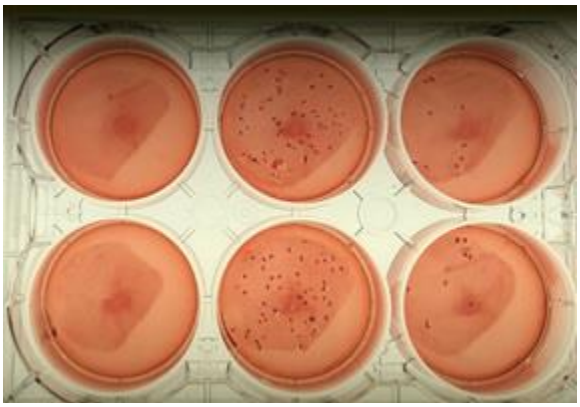


Figure 4: **Comparison of CPE for different concentrations of NHUV infected and uninfected C6/36 cells.** The uninfected C6/36 cells are round and almost confluent. The various concentrations of NHUV infected C6/36 cells show cell fusing and clumping, essentially CPE.



Control NHUV 10^{-7}dilution NHUV 10^{-8}dilution

Figure 5: Plaque assay of various concentrations of NHUV infected C6/36 cells. Plaques are well rounded and seen by day 4 of NHUV infection.

Dilution Factor	Number of plaques
10^{1}	Too many to be counted
10^{6}	46
10^{7}	31
10^{8}	9

Table 1: The number of plaques per well and the corresponding dilution factor is shown.

4.2. Peak Viral Infection of NHUV

The RT-qPCR results showed that *NS1* copy numbers reached the peak on day 4 post-infection (Figure 6). This was relatively consistent with the plaque assay results, indicating that the viral titer peaked on day 5 in the supernatants (Figure 7).

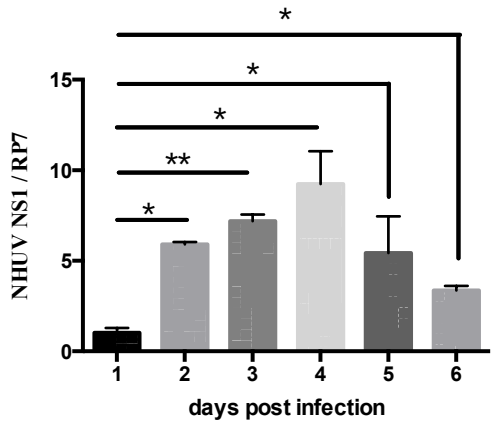


Figure 6: RT-qPCR measuring NHUV RNA replication. The copy numbers of NHUV *NS1* gene were quantified by the RT-qPCR and *Rp7* was used as the housekeeping gene internal control. This graph shows that NHUV viral replication was highest on day 4 post-infection. The data represent the results of two independent experiments and were analyzed by one-way ANOVA followed by Tukey's test (* and ** denote $p < 0.05$ and $p < 0.01$ respectively, when compared to day 1 post-infection).

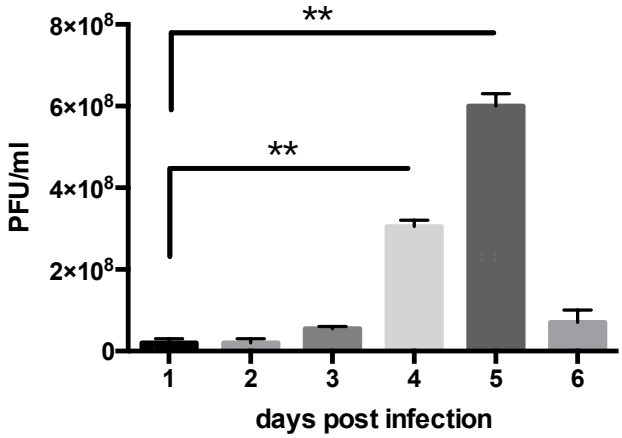


Figure 7: Plaque assay measuring infectious viral particles in the cell medium. A plaque assay was performed using the supernatant collected each day post-infection. This graph shows that the highest number of plaques was observed on days 4 and 5 post-infection. The data represent the results of two independent experiments, and were analyzed by one-way ANOVA, followed by Tukey's test (** denotes $p < 0.01$, when compared to day 1 post-infection).

4.3. Verification of the Formation of ZNHUV via RT-qPCR

ZNHUV plasmids were transfected into C6/36 cells, and the cells were inspected daily for one week. No CPE was observed in neither the negative control nor the ZNHUV

transfected group. The RT-qPCR was conducted to quantify ZIKV *E* gene using probe-based qPCR, while the NHUV *NS1* was quantified by using SYBR green-based qPCR. The qPCR results indicated that there was a similar trend between the ZIKV *E* and NHUV *NS1* gene expression. The first infection showed the highest amplification for both genes (Figure 8), which may be due to the high numbers of plasmids that were transfected into the cells initially. There was a reduction in the second and third generations compared to the first generation, but amplification started to show a trend of increase in the fourth and fifth generations. The reason could be that the new chimeric viruses need to take several generations to adapt to the cell culture before producing higher viral titers. Due to the presence of both ZIKV *E* and NHUV *NS1* even after the fifth generations, our results indicate the successful production of ZNHUV, a chimeric virus that can be potentially evaluated as a ZIKV vaccine candidate.

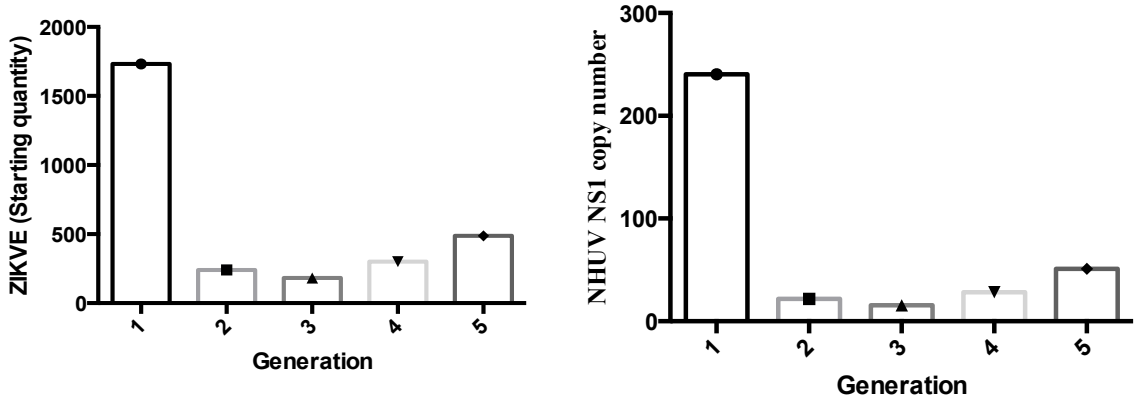


Figure 8. RT-qPCR results measuring ZIKV *E* gene and NHUV-*NS1* gene. The graph shows copy numbers of ZIKV *E* and NHUV *NS1* in the 5 generations. For the first generation, there was a higher rate of infectivity of both ZIKV and NHUV. This trend continued to decline until generation 4, where the levels of both target virus genes started to increase again. Statistical analysis was not performed because only one experiment was conducted (no repeats).

Chapter 5: Discussion

The emergence and re-emergence of human pathogenic flaviviruses is a global health concern (9) and currently, specific vaccines are not available for most of the flaviviruses. As a consequence, the development of safe and effective vaccines against pathogenic flaviviruses remains a top priority. A central problem with finding a vaccine for ZIKV, in particular, is that about 80% of the cases in patients are asymptomatic which impedes the process by which follow-up volunteers can report (12). Zika continues to be a global problem in several regions and there is a critical need for a vaccine. The primary goal of this study was to test the hypothesis of whether NHUV can be used as a backbone to generate ZIKV vaccine candidates. The Envelope (*E)* gene from ZIKV was used to replace the Envelope (*E*) of NHUV. The target plasmid product which contained the ZNHUV recombinant virus was transfected into C6/36 cells. These cells were grown for a week and the supernatant was used to re-infect five more generations consecutively. In addition to this, total RNAs from the infected cells were extracted during all five generations.

To grow the original wild-type NHUV stock, C6/36 cell lines were infected to collect the cell medium supernatant. We found that NHUV replicated with the highest concentration on day 4 post-infection (Figure 6). Thus, the ZNHUV supernatants and cells were collected on that day throughout all the five generations. The original wild-type NHUV can cause severe cytopathic effects (CPE) starting from day 2 post-infection (Figure 5). The CPE includes cell lysis and fusion. The more concentrated the viruses are, the earlier the CPE appears. Previous studies have shown that flaviviral infections can lead to both apoptosis and necrosis with an extreme level of viral load *in vivo* (19). These

viruses have the ability to activate the tumor necrosis factor α (TNF α) and other pathways leading to apoptosis (19). When ZNHUV was used to infect the C6/36 cells, there was no visible CPE. Based on physical visibility, we could not tell the difference between the ZNHUV infected C6/36 cells and the uninfected C6/36 control cells. When plaque assays were performed for ZNHUV infected cells, there was also no obvious plaque formation. These results indicate that ZNHUV either has a low concentration or has less infectivity compared to the wild type NHUV, which may be because the new chimeric ZNHUV needs to adapt to the cells by acquiring natural mutations after serial passages in the cell culture. Despite the fact that CPE was not observed in the infection with ZNHUV, the presence of the chimeric virus was confirmed using RT-qPCR to measure the presence of both ZIKV *E* and *NS1* gene of NHUV (Figure 8). The expression levels of ZIKV *E* and *NS1* were similar, with the first generation showing the maximum level of viral infection, which may be due to the high numbers of plasmids that were transfected into the C6/36 cells initially. After a brief reduction in the second and third generations, ZNHUV gradually might adapt to the cell culture and produce higher viral titers in the fifth generation. In summary, these results show the successful generation of a chimeric virus (ZNHUV) that carries ZIKV *E* in the NHUV genome backbone.

Previous studies have reported the wide-spread use of C6/36 cells in many studies *in vitro*, although it is limited due to its defectiveness in antiviral immunity (9). Furthermore, other studies have shown that results from *in vitro* studies never completely match results from *in vivo* studies (5). Compounding this, NHUV is a relatively new virus that has not yet been fully explored regarding its infectivity in mosquitoes. This study is a preliminary exploration using NHUV to generate vaccine candidates against another

important human pathogen flavivirus, ZIKV. The next step is to test if ZNHUV can infect Vero cells and evaluate if it can generate immunity against ZIKV infection in a mouse model.

ZIKV is hypothesized to have originated from Africa, then spread to Asia and the Americas (16). Those strains in the Americas have the Asian genotype, being distinct from the African strain (16). Due to this, it is essential that any vaccine developed to combat the virus has to be effective for all strains (16). In the meantime, since there are no ZIKV vaccines that have been approved, preventative measures must be taken to prevent its spread (16). For example, the mosquito vectors can be controlled by eliminating breeding sites or using insecticides and larvicides, or the rate of sexual transmission can be reduced (16). The vector control mechanism has its limitations depending on the scale. When viewed on a large-scale, vector control mechanism does not show as much promise compared to smaller-scale measures (14). In addition, it will be difficult for less developed countries to achieve effective vector control mechanisms (14). Flaviviral mutations have added to the spread of the viruses in new populations (16). This together with increased globalization and urbanization are likely reasons for the continuous emergence and reemergence of ZIKV and other flaviviruses (16). This is because the aforementioned processes involve an increase in temperatures, human movement, an increase in goods transportation, and also population densities (14).

In conclusion, the results presented in this thesis provide some preliminary but essential methodological understanding of the infectivity of NHUV. The results also indicate that a chimeric ZNHUV virus can be successfully developed. Ultimately, this

study lays a foundation for the further development of safe and effective vaccines for ZIKV, using the NHUV genome as a backbone.

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