CHIKUNGUNYA VIRUS GLYCOPROTEINS MEDIATE VIRAL ENTRY AND CELLULAR FUSION

by

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BS, University of Pittsburgh, 2010

Submitted to the Graduate Faculty of
Graduate School of Public Health in partial fulfillment
of the requirements for the degree of

Master of Science

University of Pittsburgh

2012
UNIVERSITY OF PITTSBURGH

Graduate School of Public Health

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The Chikungunya virus (CHIKV) has currently been identified in over 40 countries and in 2008 was listed as a US National Institute of Allergy and Infectious Diseases (NIAID) category C priority pathogen. Outbreaks of the virus have been documented as early as 1779 and frequent outbreaks have been reported through 1960-2003, most notably in Reunion Island, a French overseas department in the Indian Ocean. Out of a total population of 785,000 in Reunion Island, 300,000 cases were reported including a total of 237 deaths. Numerous aspects of the viral life cycle are unknown, with no current vaccine the implementation of more research and dissemination of more knowledge is of great public health importance. A CHIKV construct was synthesized by Genewiz, containing CHIKV structural proteins in pcDNA 3.1. This construct was used to create CHIKV pseudo-viral particles with a luciferase based reporter. The pseudo-virus was used to survey many cell lines for permissivity to infection. This construct was also used to create 3 other constructs containing CHIKV E1, E2, and E3 individually. These constructs were used individually and in combination with each other to create pseudo-viruses for cellular infection. The cell-cell fusion capabilities of the full CHIKV construct along with the individual envelope proteins were also tested in a Cre-Lox system.
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The famous saying always goes there is no “I” in “TEAM”. This phrase couldn’t be truer when it comes to the dissemination of great knowledge and the pursuit of fascinating research. I would like to first thank Dr. Tianyi Wang for being a fantastic mentor, teacher, role model, and friend. Without his guidance, wisdom, and patience none of my research would have been possible. I would also like to thank my committee members, Dr. Amy Hartman and Dr. William Klimstra, for dedicating their time and effort to me and lending me their sound advice. Finally, I would like to thank my lab members Dr. Shufeng Liu and Aram Lee for their assistance and guidance during my times of need in the lab.
1. INTRODUCTION

The Chikungunya virus commonly referred to as CHIKV is a word derived from the Kimakonde language of Mozambique meaning ‘to walk bent over’ (Sourisseau, M., et al.). Due to an inflammatory response seen in victims joints they often assume a bent posture and find it hard to maneuver their limbs (almost to a point of paralysis). The virus is a positive-sense single stranded RNA virus belonging to the genus of Alphavirus and the family of Togaviridae. Outbreaks of the virus have been documented as early as 1779 and frequent outbreaks have been reported through 1960-2003 in areas of Malaysia, Indonesia, Cambodia, Vietnam, Myanmar, Pakistan, Burma, and Thailand. The most notable outbreak was seen in Reunion Island in France through 2005 and 2006 where about one-third of the entire country's population was infected by CHIKV. Out of a total population of 785,000 people 300,000 cases were reported including a total of 237 deaths. CHIKV has currently been documented in over 40 countries and is listed as a US National Institute of Allergy and Infectious Diseases (NIAID) category C priority pathogen. (Sourisseau, M., et al.)
1.1 EPIDEMIOLOGY

CHIKV had its first recorded epidemic in Tanzania in 1952 and was first isolated after this event (Her, Z., et al). Many infections have been reported in Vietnam, Burma, Cambodia, Sri Lanka and the Philippines. Some sequencing data suggests that CHIKV originated in Africa and was introduced later to many parts of Asia. Many epidemics of CHIKV were also reported in the Philippines between the 1950’s and 1960’s, and many outbreaks were reported in India from the 1990’s to early 2000’s (Sudeep, A.B et al). One of these outbreaks was seen in Kinshasa between 1999-2000 infecting an estimated 50,000 people. The virus was first isolated in 1963 in Calcutta, India (Pialoux et al). Since 2005 it is estimated that there have been more than 1,400,000 cases in India. Between 2005 and 2006 it has been estimated that there are approximately 1,400,000 to 6,500,000 cases in India (Sourisseau, M., et al.). Other cases have been reported in Germany, Norway, China, Italy, and Switzerland. Many of the large epidemics seen are typically centered on seasons with heavy rain. Seasons with large amounts of rain increase the density of the vector population; therefore, increasing human contact with the vector. Malaysia was hit with its first CHIKV epidemic in 1998, which was brought in from migrant workers. Many epidemics of CHIKV have also been plagued by infections with not only CHIKV, but also with yellow fever, dengue virus, and Plasmodium falciparum.

The scariest epidemic of CHIKV was seen in Reunion Island in 2006 with 300,000 cases reported in a population just under 800,000 (Bonn, D.) (Schuffenecker, I., et al). The rapid spreading of the virus to so many people was associated with a change in mosquito species from Aedes aegypti to Aedes albopictus due to an alanine to valine at position 226 mutation of the E1 envelope protein of the virus (D'Ortenzio, E., et al). It has been speculated by some that the virus
could have been spread by other means than a mosquito vector, such as a respiratory spreading (although no data has been shown that the virus actively infects lung cells to date). A possible respiratory infection may also explain the over 200 deaths associated with the Reunion Island outbreak (which is typically non-fatal). Another proposed theory on the Reunion Island outbreak is that the virus entered the island into a population with no immunity. The highly sensitive population was infected at an increasingly high rate due to the large abundance of the *Ae. albopictus* mosquito (Sourisseau, M., et al.). Interestingly during the same outbreak of CHIKV out of 35 pregnant women who were infected during delivery, 30 of these women delivered an infected child. (Pialoux et al). Due to this recent switch in mosquito species to *Ae. albopictus* many reports of infection have been documented in the USA, Europe, and numerous South East Asian countries (Gibney, K.B., et al). This included a recent outbreak in 2007 in Kerala, India that infected 70,731 individuals, which was directly associated with the A226V mutation (Kumar, N.P., et al). These cases have mainly been linked to infected individuals who either travel or work internationally. (Lo Presti, A., et al) (Petersen, L.R et al) (Santhosh, S.R., et al) (Schuffenecker, I., et al) (Sudeep, A.B et al) (Sourisseau, M., et al.)

1.2 TRANSMISSION

CHIKV is transmitted by the Aedes species of mosquitoes. The virus follows two forms of transmission, an urban cycle and a sylvatic cycle. The urban cycle uses humans as a main reservoir. In this cycle the mosquito bites a human infected with the Chikungunya virus, which is then passed on to another human when they are bitten by the infected mosquito. In the sylvatic cycle animals including; monkeys, rodents, birds, and a few unidentified vertebrates
serve as reservoirs while the Aedes mosquitoes still act as vectors and transmit the disease to humans. This cycle works similarly to the urban cycle, but instead of the mosquito becoming infected by a human reservoir, they are infected by an animal reservoir. The infected mosquito can then infect a human with the virus, which can reinitiate the urban cycle or continue the sylvatic cycle. (Petersen, L.R et al) (Singh, S.K.)

Even though some non-human vertebrates have been shown to carry the virus, humans have been the only documented reservoirs to be present during epidemics. In fact the sylvatic cycle of the virus has not yet been reported in Asia, but only Africa. (Gibney, K.B., et al.)

The main mosquito vector during the early outbreaks of CHIKV was the *Ae. aegypti*. During the Reunion Island outbreak a mutation was seen changing this to the *Ae. albopictus* mosquito. The A226V mutation not only caused increased fitness in *Ae. albopictus*, but also caused an increase in transmissibility and a reduction of infectivity in *Ae. aegypti*. The *Ae. albopictus* mosquito has seen a recent expansion in territory from South East Asia into Madagascar, the Indian Ocean, Africa, and temperate areas of Southern Europe and the U.S.A. (Jain, M et al.) (Kononchik, J.P., et al.)
1.3 VIRAL GENOME AND STRUCTURE

The Chikungunya virus belongs to the genus of Alphavirus in the family of Togaviridae. It has a single stranded RNA genome approximately 11.8 Kb in length. As with other Alphaviruses CHIKV is very small having an icosahedral shape, 60-70 nm capsid, and a phospholipid envelope (Gibney, K.B., et al.) (Leung, J.Y., et al.). It is currently proposed that CHIKV follows similar methods of replication as other Alphaviruses.

1.3.1 STRUCTURE

Chikungunya virus gains its envelope from the host after viral shedding. The envelope is a phospholipid biliayer mainly comprised of the lipids contained within the hosts own plasma.

**Figure 1. A depiction of the CHIKV replication.** The urban cycle (left) shows the infection cycle from human, to mosquito, and back to human. The sylvatic cycle (right) shows the infection cycle from a non-human mammal, to mosquito, and then to human.
membrane. With CHIKV seeming to be very closely related with other Alphavirus structures it is most probable that the thickness of the virion envelope is similar to the Sindbis virus (SIN). SINs envelope has a thickness of approximately 4.8 nm and is centered at a radius of 23.2 nm (Strauss, J.H. et al.). It was proposed that CHIKV followed a similar structure of other Alphaviruses with 240 copies of E1 (approximately 63kDa in SIN) and E2 (approximately 59 kDa in SIN) embedded into the outer membrane (Anthony, R.P., and D. Brown). This was later confirmed by x-ray crystallography (Voss, J.E., et al.) showing E1 and E2 forming 80 spikes arranged in a T = 4 icosahedral structure. E1 and E2 are anchored in the membrane by membrane-spanning anchors found in their C-terminus. Cross-linking studies done on other Alphaviruses suggest that the envelope trimers are held together by E1-E1 binding. The trimer spikes stalk is formed by an anti-clockwise twisting of the three E1-E2 heterodimers. The tip of each heterodimer has an E1 and E2 separation, and the spikes extend approximately 34 nm from the envelope (giving the virion its total diameter). (Strauss, J.H. et al.) (Anthony, R.P., and D. Brown) (Kononchik, J.P., et al.)

1.3.2 GENOME AND REPLICATION

CHIKV contains two open reading frames (ORFs), a 5’ cap structure and a 3’ poly A tail. The first ORF is responsible for producing the non-structural proteins with two polyprotein precursors of nsP1, nsP2, nsP3, and nsP4. The read through codon is responsible for making a major product of nsP123, and a minor product of nsP1234. The second ORF is responsible for producing the viral structural proteins: the capsid proteins; envelope glycoproteins E1, E2, and E3 and an additional protein, 6K. The viral envelope protein E3 appears to protect E1 from fusogenic conformational changes during egress, and is a secreted protein. Envelope protein E2
is postulated to be responsible for viral attachment. E1 may be responsible for promoting the release of the viral nucleocapsid (Kuo, S.C., et al). Two-hundred-and-forty copies of E1 and E2 form heterodimers that are imbedded into the CHIKV viral membrane (Tsetsarkin, K.A. et al). Together these glycoproteins would appear to be the main drivers in attachment to the host cell. The E2 and E1 heterodimers have been shown to cause viral membrane fusion by a cholesterol dependent mechanism (Kuo, S.C., et al). The 6K (approximately 6000 Da) protein appears to potentially have multiple roles in glycoprotein processing, cell permeabilization, and viral budding (Jose, J., et al.) (Kuo, S.C., et al) (Nyas, K.P., et al) (Singh, S.K.) (Kononchik, J.P., et al.)

Figure 2. Rendition of the CHIKV Genome. The genome consists of two open reading frames, creating the non-structural protein 1-4 (nsP1, nsP2, nsP3, and nsP4) and the structural protein (C-Capsid, Envelope proteins 1-3, and glycoprotein 6K). The virus genome also has a 5’cap and 3’ poly A tail.

1.4 VIRAL LIFE CYCLE

The life cycle of the CHIKV virus is currently still being researched. It is proposed that CHIKV follows a similar life cycle to other Alphavirus and Togaviridae such as Sindbis virus, Semliki Forest virus, and Ross river virus (Singh, S.K.) (Sourisseau, M., et al.). The life cycle
starts by the virus attaching to the host cell by an unknown receptor. It is currently proposed that virus uses the E2 viral receptor for attachment to the host although the actual host binding receptor is unknown. With the broad spectrum of virally infectable cell lines, it would appear the receptor is something common between a multitude of cell lines (Sourisseau, M., et al.). The virus is then taken up by some form of mediated endocytosis (most likely clathrin coated) (Liljestrom, P., et al.). A low endosomal pH would then expose a fusion peptide to fuse the viral envelope with the host membrane and release the nucleocapsid into the cells cytoplasm. The nsP123 and nsP1-4 would be translated from the viral genome, then nsP123 would bring nsP1234 along with other host factors to produce a replication complex (RC). The RC would then produce full length negative strand RNA. When nsP123 has a sufficient concentration it would be cleaved into nsP1, nsP2, nsP3, and nsP4, and, alongside host proteins, act as a plus strand replicase to produce a 26s RNA strand. A promoter would then initiate the transcription of 26s sub-genomic positive RNA. The structural polyprotein then would most likely be cleaved into the individual structural proteins. A nucleocapsid protein would be produced and other glycoproteins would be sent to the ER for processing. The E2 and E3 proteins appear to be processed as a single polypeptide until cleaved by a furin-like protease, and have been shown to be semi-unstable alone at low pH (Jose, J., et al.). The E2 precursor is commonly known as PE2 or p62. The E2/E3 polyprotein is critical for preventing E1 from assuming a fusogenic conformation during low pH stages in egress. The preprocessed form of E3 and E2 is commonly referred to as the p62 structure (Voss, J.E., et al). The complex of proteins would then move through the Golgi and the virion would start to be assembled in the cytoplasm. The virus would then bud through the host membrane. E1 and E2 also appear to form a bilayer consisting of
The pathogenesis of CHIKV occurs with the initial bite of an infected mosquito. From here there would be some form of receptor mediated endocytosis into susceptible cells and production of the virus. (Her, Z., et al) A recent mouse model showed that the virus seems to first target and
replicate in the liver, and then target muscles, joints, and skin. The virus has been shown to actively replicate in lymphoid and myeloid cells, and induce apoptosis in Hela cells and primary fibroblasts. Cholesterol depletion has been shown to lower infectivity by about 65%. (Singh, S.K.) The virus mainly seems to target the cells in muscles, especially joints, primarily bone and joints associated with connective tissue and skeletal muscles. CHIKV infection usually clears in roughly 1 week, possibly hinting the innate immune system as the main combater. Since CHIKV mainly seems to be an acute disease the chronic aspects have been poorly studied, although many people complain of myalgia years after infection. (Her, Z., et al) (Singh, S.K.) (Gunn, B.M., et al.)

1.6 SYMPTOMS AND DIAGNOSIS OF CHIKV INFECTION

CHIKV infection has a silent incubation period between 2-5 days (Birendra et al) (Pialoux et al), and is associated with very high fever over 100°C. Initial symptoms appear to include headache, throat discomfort, abdominal pain, and constipation. Encephalitis is not a common symptom seen in CHIKV as in other Alphaviruses (Levine B., et al.), and it has not been seen that CHIKV is able to actively infect brain cells. Severe arthralgia is the main symptom with victims having mild to very severe joint pain and swelling. The arthralgia appears to be extremely intense affecting mainly the ankles, wrists, and phalanges. (Petersen, L.R et al) Arthralgia in infected patients seems to be very erratic and crippling. (Pialoux et al). Currently the mechanism associated with joint pain is unknown. It is speculated that the inflammation could be caused by an immune reaction the body uses to combat the virus. Considering symptoms seem to persist even after the clearing of the virus, it would be more probable to assume it is some form of auto
immune response. (Pialoux et al) Some other documented symptoms include chronic arthralgia, conjunctivitis, enlarged lymph nodes, and rashes on the body trunk and ear lobes. One patient was also recorded with having facial swelling and itching, which was associated with an oral candidiasis associated with CHIKV (Kumar, J.C., et al). Patients displaying symptoms of CHIKV are often misdiagnosed with dengue virus, although hemorrhagic fever has been reported in Thailand associated with CHIKV infection. Most of the symptoms of CHIKV appear to generally resolve themselves within about a week, but joint pain and swelling have been noted to last years. (Kuo, S.C., et al) (Petersen, L.R et al) (Sudeep, A.B et al) (Gunn, B.M., et al.)

Currently there are a few diagnostic tests which are used in order to identify a CHIKV infected patient. These include viral isolation, RT-PCR, and ELISA. The best method of detection involves viral isolation, exposing cell lines to samples of the patients blood and identifying responses to CHIKV. In the United States this method of viral isolation; however, must be performed in a BSL 3 laboratory, and takes approximately 2 weeks. RT-PCR is very good at detecting virus from the day of infection to day 7, and takes only a few days to perform. Serological tests such as ELISA are very easy to perform and can detect IgM about 2 days after infection. ELISA unfortunately tends to show many false positives due to cross reactivity with many other arboviruses. (Kumar, J.C., et al) (Pialoux et al) (Sudeep, A.B)

1.7 TREATMENT AND PREVENTION

The Chikungunya virus currently has no commercially available vaccine or treatment, leaving protection from mosquitoes as the main method in preventing viral spreading (Reiter, P. et al).
One of the first direct methods in preventing mosquito contact would be to remove mosquitoes from any human inhabited areas (such as cities and homes). Mosquitoes breed in areas with small and/or large pools of stagnant water. Many people can protect their homes by removing these pools of stagnant water from around their yard and neighborhood. If people are aware of areas with high mosquito populations they can wear protective clothing. Protective clothing would be anything having a large amount of skin coverage that is not able to be penetrated by the mosquito's bite. A type of insecticide known as pyrethoids can also be used to treat clothing. This specific type of insecticide can also be vaporized, which acts as a mosquito repellent. Other commercially available repellents can also be purchased, which include N,N-diethyle-meta-toluamide (DEET), icardin, and p-menthane-3 8-diol (PMD). (Reiter, P. et al) Many large government agencies, such as the CDC in the U.S., track the progression of large mosquito populations and attempt to control the mosquitoes migrations into urban areas. The methods of countering the mosquito population can be very labor intensive and expensive. This is due to the constant adaptation of mosquitoes to resist insecticides, and their overall large population. (Pialoux et al)

With no currently available vaccine the best practice for treating CHIKV is to treat each individual symptom to ensure a patients comfort. The methods of treatment typically include the distribution of analgesics and non-steroidal anti-inflammatory drugs (NSAIDs). A few vaccine models are currently being test. These vaccines include a virus-like particle (VLP) vaccine which showed protection in monkeys (Akahata, W., et al) and a DNA vaccine which is designed based on CHIKV capsid an envelope sequences (Birendra et al). The US Army Medical Research Institute have also been testing a live vaccine based on the CHIKV strain 15561. The
virus was attenuated by passaging through MRC-5 cells, and has started human testing in a phase III trial. Chikungunya infected patients fortunately seem to give a long lasting immune response to CHIKV after infection.

New migration patterns of the Ae. Albopictus mosquitoes create a frighteningly real threat that the CHIKV virus may be coming to the U.S. With the high infectivity seen in the Reunion Island outbreak the introduction of the virus into the U.S. could be detrimental not only to the health of the people, but to the economical status of the areas infected. With a quickly spreading outbreak, the workforce is diminished very quickly leaving no one to perform the daily jobs that keep us going every day. As an example imagine there was a very large outbreak of CHIKV leaving a large area unable to work. It is possible the people in this area could work for a large power company. With no one to run the machinery in the power plant, energy could not be supplied to local business. With the new A226V mutation the virus could spread very rapidly and incapacitate an extremely large workforce for weeks to months. This would drastically impact the economy, and unfortunately with unknown future mutations, may become a more deadly infection. This would highlight the need to an increase in understanding of the virus and implementing more research in vaccine and treatment developments.
2.0  AIM 1 - INVESTIGATING THE CELLULAR TROPISM OF CHIKV USING PSEUDO-TYPED VIRUS

2.1  AIM 1.1 - CREATING A CHIKUNGUNYA PSEUDO-VIRUS SYSTEM

Currently, in the U.S., the Chikungunya virus is studied at a BSL 3 safety level. With the confines of working in a BSL 2+ laboratory it was imperative that we find a suitable system to study CHIKV under these conditions. A pseudo-viral system was used by creating a Chikungunya virus lacking its non-structural proteins, and keeping its structural proteins. Pseudo-viruses have been shown to be extremely useful in the fields of scientific advancements and previous reports showed the early African strain 37997 as being a good candidate for pseudo-viral use (Salvador et al), we chose this strain as our template (http://www.ncbi.nlm.nih.gov/nuccore/AY726732.1). The Chikungunya virus construct was crafted using Vector NTI and sent to the company Genewiz for synthesis. The construct contained the CHIKV viral structural proteins from the capsid to the E1 protein, inside the multiple cloning site (MCS) of pcDNA3.1 expression plasmid (Figure 4).
Figure 4. Synthesized CHIKV 37997 expression plasmid. CHIKV structural proteins were placed inside the multiple cloning site of pcDNA 3.1, which were cloned by Genewiz. The plasmid contains CHIKV structural proteins core, E3, E2, E1, and 6K.

2.2 AIM 1.2 - CHIKUNGUNYA PSEUDO-VIRUS

In order to assess the cellular tropism of the virus a stable pseudo-virus was created using the plasmid created by Genewiz (pCHIKV 37997). A transfection was performed on 293T LentiX cells containing the plasmids pTrip Luciferase, ΔR8.2 (http://www.addgene.org/12263/), and pCHIKV 37997. This would create a viral particle that has a HIV core structure (from the ΔR8.2
construct), expressing CHIKV viral envelope proteins (from the pCHIKV 37997 construct), and is able to be detected by the internalized luciferase construct (pTrip Luciferase).

### 2.2.1 AIM 1.2.1 - CELLULAR TRANSFECTION

In order to perform the transfection 293T LenX cells were first grown in DMEM (Dulbecco's Modified Eagles Medium) complete media containing 10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin, and 1% non-essential amino acids. Cells were grown in a 10cm dish to confluence of about 70%. A transfection mixture was then made by placing pCHIKV 37997, pTrip Luciferase, and ΔR8.2 in Optimem at a 1:1 ratio (Figure 5). Polyethylenimine (PEI) was added to the Optimem at a concentration double to the amount of plasmid DNA added. For example if there was 5ug of DNA added, then 10ug of PEI was added. The addition of PEI positively charges the added plasmid DNA and allows it to better target the anionic surface of the cells for better uptake. The Optimem mixture was allowed to set for about 30 minutes. While the Optimem was setting pre-warmed DMEM containing only 10% FBS was added to the 293T LenX cells, after the removal of the old complete DMEM. The Optimem mixture containing DNA and PEI was added dropwise to the 293T cells. This transfection mixture was allowed to sit on the cells for 5 hours. After 5 hours the media was changed to pre-warmed complete DMEM. Once 24 hours passed the old DMEM was removed and replaced with fresh complete DMEM. The pseudo-virus was then harvested after 48 hours.
2.2.2 AIM 1.2.2 - HARVESTING PSEUDO-VIRUS

To harvest the virus the supernatant was removed and spun in a centrifuge for 15000xg for 5 minutes. This ensures that most of the dead cells and/or cellular debris are removed from the viral containing media. After centrifugation the media was removed from the cell pellet and run through a 0.45 µm filter syringe into a clean microfuge tube. Virus that was not used for infection immediately was aliquoted into 2mL microfuge tubes and placed at a -80° freezer.

![Figure 5. Visual representation of pseudo-viral transfection method.](image)

Plasmids ΔR8.2, pTrip Luciferase, and pCHIKV 37997, were co transfected into 293T LentiX cells in order to produce a HIV based pseudo-virus expressing CHIKV envelope glycoproteins.

2.2.3 AIM 1.2.3 - PSEUDO-VIRAL INFECTION

In order to ensure the virus was actively able to infect cells a trial infection was performed on 293T cells. 293T cells were plated in a 48 well plate to a confluencey of 70%. The freshly collected CHIKV pseudo-virus, along with a VSV-G positive control, pcDNA3.1 blank, and a
pseudo-virus containing only pTrip-Luciferase and pCHIKV 37997 were used to infect the 293T cells. The pseudo-virus containing only pTrip Luciferase and pCHIKV 37997 was used as a control, considering the pCHIKV 37997 construct still contained the capsid proteins it needed to be verified that the capsid wasn't creating viral-like particles containing an encased pTrip-Luciferase plasmid. To infect the cells the collected virus was added to the cells after the removal of the media. Before addition of virus the chemical polybrene was added to the virus at a concentration of 4 ug/uL in order to increase the viral infectivity.

Figure 6. Graphical Representation of Pseudo-Viral Infection and Cellular Lysis to Luciferase Reading. Cells were initially infected with CHIKV pseudo-virus. After 48 hours a 1x passive lysis buffer was added to the cells to induce lysis. Cells were shaken on an electronic shaker for approximately 15 minutes with the lysis buffer. After 15 minutes the cell lysate was placed in a luminometer white plate and read in a luminometer.
CHIKV pseudo-virus was made in 293T cells by transfecting the cells with the synthesized CHIKV construct, pTrip Luciferase, and ΔR8.2. Pseudo-virus was used to infect an initial round of 293T cells. A positive control was made using VSV-G instead of CHIKV. Two negative controls were made, one containing pcDNA3.1 and the other containing only the CHIKV construct and pTripLuciferase. The pseudo-viral infection was allowed to go for 5 hours on the cells and was then replaced with pre-warmed fresh complete DMEM media. The cells were then allowed to incubate for 48 hours. After 48 hours the cells were lysed with 1x passive lysis buffer. After lysis a luciferase activating reagent (LAR) was added to the lysates and the lysates were read on a luminometer (Figure 6). The readings suggested that the CHIKV pseudo-virus was in fact infectious to 293T cells compared the positive and negative controls. This data provided proof that this pseudo-virus could be used in the subsequent screening assays (Figure 7).
To this date the amount of survey data present on the Chikungunya virus seems very limited. Knowing which cells lines the virus actively infects could lead to a better understanding of the human pathogenesis, and could lead to the use of certain cells lines for future studies.

In order to evaluate which cells lines could be infected CHIKV pseudo-virus was created, along with the VSV-G positive control virus, and the two negative controls containing either pcDNA 3.1 or pCHIKV37997 and pTrip Luciferase. These pseudo-viruses were created in the same fashion as in AIM 1.2. Human epithelial kidney 293T (HEK 293T) cells (originally derived from HEK 293 cells by the addition of SV40 large T-antigen), Huh 7.5.1 hepatoma cells (derived from Huh 7.5 cells lacking RIG-1), human brain microvascular endothelial cells (HBMECs) (isolated from cortical tissue, highly specialized and responsible for blood-brain barrier formation), Verda Reno (VERO) African green monkey kidney cells, A549 adenocarcinomic human alveolar basal epithelial cells (cultured from the removal of cancerous lung tissue), Caco-2 human epithelial colorectal adenocarcinoma cells (cultured cells resemble enterocytes lining the small intestine), C8 mouse macrophage cells, Jurkat T lymphocyte cells (immortalized IL-2 producing T-cell derived from a 14 year old boy), H9 T-cells (derivative of Hut 78 T-cells derived from peripheral blood of a Sezary syndrome patient), CEM-SS T4-lymphoblast cells, and C6/36 mosquito cells (derived from Ae.Albopictus mosquito larval tissue) were all of the cell types infected with the CHIKV pseudo-virus. The adherent cell lines were infected in the same manner as the 293T cells. Suspension cell lines (C8, Jurkat, H9, and CEM-SS) were infected by spin infection.
2.3.1 AIM 1.3.1 - Pseudo-Viral Spin Infection

Before cells were infected a centrifuge was allowed to heat to 33°C. After the centrifuge was allowed to heat cells were suspension cells were pelleted down and resuspended in viral containing media. The suspension cells containing virus were placed in 24-well plates and allowed to spin at low speeds (about 2000xg) for an hour. After 1 hour the suspension cells were pelleted down in clean microfuge tubes and re-suspended in fresh complete media. These were then transferred back into the appropriate wells. After infection the cells were allowed to grow for 48 hours. After 48 hours the cells were pelleted down and lysed with 1x passive lysis buffer (as done before with adherent cells) and read in a luminometer (Figure 6).
Table 1. CHIKV Pseudo-viral Infection Survey of Multiple Cell Lines.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Infectable (+)/Non-Infectable (-)</th>
<th>Relative Light Units (RLUs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK 293T</td>
<td>+</td>
<td>*****</td>
</tr>
<tr>
<td>Huh 7.5.1</td>
<td>+</td>
<td>*****</td>
</tr>
<tr>
<td>HBMEC</td>
<td>+</td>
<td>****</td>
</tr>
<tr>
<td>VERO</td>
<td>+</td>
<td>***</td>
</tr>
<tr>
<td>A549</td>
<td>+</td>
<td>**</td>
</tr>
<tr>
<td>CaCO2</td>
<td>+</td>
<td>**</td>
</tr>
<tr>
<td>C8</td>
<td>+</td>
<td>*</td>
</tr>
<tr>
<td>Jurkat</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>H9</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>CEM-SS</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>C6/36</td>
<td>-</td>
<td>*</td>
</tr>
</tbody>
</table>

Using the same pseudo-typing method to create pseudo-virus in figure 1, CHIKV pseudo-virus was used to infect numerous cell lines. RLU s are a depiction of the averages from 3 individual trials in 3 independent experiments. * Under 200 RLU s, ** 1000-10,000 RLU s, *** 10,001-20,000 RLU s, **** 50,000 – 1,000,000 RLU s, ***** 1,000,001 – 2,000,000 RLU s

2.3.2 AIM 1.3.2 - PSEUDO-VIRAL INFECTION RESULTS

After adherent and suspension cells had been read on the luminometer, luciferase data was compiled. Cell lines with readings under 200 relative light units (RLUs), were deemed not infectious in comparison with the blank reading. Readings over 1,000 RLUs were deemed as infectious (Table 1). In order to accurately interpret the results, all positive and negative readings were also displayed graphically (Figures 8, 9, & 10).
Figure 8 A-F. Positive Results of CHIKV Pseudo-viral Infected Cells with Positive and Negative Controls. Cell lines from table 1 that were shown to be infectable are shown here displayed with the appropriate controls as in figure 6. Each individual result is the product of 3 individual trials performed in 3 independent experiments.

Figure 9 A-C. Negative Results of CHIKV Pseudo-viral Infected Cells with Positive and Negative Controls. Cell lines from Table 1 that were shown to not be infectable are shown here displayed with the appropriate controls as in figure 6. Each individual result is the product of 3 individual trials performed in 3 independent experiments.
The data shows that all cells except the cells of the T-cell lineage and the C6/36 mosquito cell line were infectious to the CHIKV pseudo-virus (Table 1) (Figures 8, 9, & 10). The non-infectious nature of CHIKV to cells of the T-cell lineage seems consistent with results seen with Ross river virus and Semliki forest virus (La Linn, M., et al.) (Strang, B.L., et al.). This data
would seem consistent with some other cellular infections that have been performed (Tang, B.L et al) (Sourisseau, M., et al.). The C6/36 cell line most probably was non-infectious due to the mosquito cell line machinery not having the correct proteins to actively produce the luciferase protein. This theory is shown to be proven further by the fact that previous data suggests C6/36 to be highly infectable (Tsetsarkin, K.A. et al). Although not performed in these trials it would be possible to test this theory by directly transfecting the luciferase plasmid into C6/36 cells and attempt luciferase detection. Interestingly the HBMEC cell line, a mimic of the human blood brain barrier, was highly infectable. Many infected patients of CHIKV have the complaint of acute and chronic migraines, along with dizziness and many other symptoms associated with brain trauma. The infection of the HBMEC cells may be a new lead to the possible infection of areas of the human anatomy associated with these symptoms. Mouse macrophage C8 cells seem consitant with other infection data showing CHIKV is able to infect human primary macrophages (Sourisseau, M., et al.). The infection of the A549 alveolar cells may also hint to a probable mechanism that was presented in the Reunion Island outbreak. It was speculated that the Reunion Island outbreak spread so fast that another mechanism besides a bite from a vector carrier may be associated. It was presented that a possible air borne mechanism could be the causation, although the mechanism of action was unknown. The lung cell infectivity of the A549 cells could provide more evidence to this new mechanism, although one article did show a contradiction to A549 infectivity (Sourisseau, M., et al.).
3.0 AIM 2 - INVESTIGATING THE CONTRIBUTION OF INDIVIDUAL VIRAL PROTEINS IN CHIKV ENTRY BY TESTING THEIR ABILITY IN PACKAGING PSEUDO-VIRUS AND MEDIATING CELL-CELL FUSION

3.1 AIM 2.1 - DEVELOPMENT OF PLASMIDS EXPRESSING INDIVIDUAL CHIKV STRUCTURAL PROTEINS

After seeing that the CHIKV pseudo-virus was not only able to infect 293T cells, but a number of other cell lines, we wanted to look at each individual viral protein and its role in viral entry. In order to do this three separate proteins were developed to express the CHIKV envelope proteins E1, E2, and E3 individually.

A method of PCR cloning was used in order to manufacture each individual structural protein into a pcDNA 4 expression plasmid. Forward and reverse primers were created (Figure 12) in order to amplify the structural proteins E1, E2, and E3 from the synthesized CHIKV 37997 plasmid. In order to simplify the process the E1 envelope proteins will be discussed in detail, but the E2 and E3 envelope proteins were created in the exact same fashion.
**Figure 11. Simplified Representation of the pCHIKV 37793.** In order to simplify the representation of the steps of the PCR process the actual Vector NTI plasmid (left) will be simplified to the other plasmid seen (right). A similar representation will be done for plasmids when specified.

**Figure 12. Simplified Drawing of Where Each Individual Primer was Designed to Amplify.** Forward and reverse primers were designed to amplify the regions of E3, E2, and E1 of the pCHIKV37997 plasmid. E3 and its primers are designated by red, E2 and its primers are designated by blue, and E1 and its primers are designated by green.
The plasmid pcDNA4 and the PCR amplified CHIKV envelopes were digested with restriction enzymes BamH1 and Xho1. The digestion left the pcDNA4 and PCR amplified envelopes with sticky ends due to the enzyme cutting.

After pcDNA4 and the amplified CHIKV envelopes had been digested as depicted in Figure 14 the products were ligated together with T4 DNA ligase. The pcDNA4 was ligated with either E3, E2, or E1.

Forward and reverse primers were developed for the E1 structural protein. A standard 50µL PCR reaction was set up including the synthesized CHIKV 37997, the forward and reverse primers, dNTPs, Phusion polymerase buffer, and Phusion polymerase. The reaction was placed into a PCR machine with an initial denaturing cycle at 98°C for 30s. A denaturing step of 98°C
for 10s, an annealing temperature of 55°C for 15s, and an extension cycle of 72°C for 30s per 1kb was repeated for 35 cycles. A final extension was performed at 72°C for 10 min. After the PCR reaction was finished the product was run on a 8% agarose gel and gel extracted. After gel extraction the product and pcDNA4 (http://tools.invitrogen.com/content/sfs/vectors/pcdna4tomychis.pdf) was digested at 4°C overnight with BamH1 and Xho1 (Figure 13). The next day the two digested products were gel extracted and ligated using DNA ligase (Figure 14). The ligated products were transformed into E.Coli and onto LB agar plates with ampicillin. Colonies were picked from each plate and grown in ampicillin LB broth. After 24 hours of growth the plasmids DNA was extracted from the E.Coli using a mini-prep kit (Figure 15).

Figure 15. Initial Steps of the E. Coli Transformation Performed to Amplify the Newly Cloned CHIKV Plasmids. After the individual CHIKV envelope proteins had been ligated into the pcDNA4 they were added to 2 mL microfuge tube containing DI water, KCM buffer, and Top10 E. Coli. The transformation mixture was then spread onto an ampicillin resistant LB agar plate. A single colony was plucked from the agar plate after about 24 hours and this colony was amplified in ampicillin resistant LB broth containing tube. A miniprep was performed on this amplified E. Coli colony using a plasmid mini kit (Omega Bio-Tech #D6943-02).
The purified plasmid DNA was then sent for sequencing to verify the insert had been uptaken by the pcDNA 4 vector. Once the plasmids had been verified by sequencing, the end result was 3 individual plasmids expressing E1, E2, and E3 respectively (Figure 16 & 17).

Figure 16. Visual breakdown of individual glycoproteins used to create envelope protein plasmids. The glycoproteins E3, E2, and E1 were individually amplified through PCR in order to be cloned into a pcDNA 4 expression vector.
Figure 17. Vector NTI Map of pcDNA4 Plasmids Containing E3, E2, or E1. After sequencing data returned for the newly synthesized CHIKV plasmids, Vector NTI maps were created in order to accurately display a graphical representation of all CHIKV plasmids.

3.2 AIM 2.2 - PSEUDO-VIRAL INFECTIONS OF HEK 293T CELLS BY INDIVIDUAL CHIKV GLYCOPROTEIN

The CHIKV plasmids containing E3, E2, and E1 in pcDNA 4 were used to infect HEK 293T cells in order to assess each of their individual roles in viral infection. Pseudo-virus was made as done before using each of the individual envelope proteins, along with a pcDNA 3.1 blank,
VSV-G positive control, and a CHIKV pseudo-viral positive control Figure 18). As before the plasmids were transfected into 293T LentiX cells, and harvested 48 hours after transfection.

![Diagram of Transfection of 293T Cells to Create Pseudo-Virus Containing Either E3, E2, or E1 Plasmids.](image)

The virus was then used to infect 293T cells within a 48-well plate. After a 5 hour infection the media was changed with fresh pre-warmed complete DMEM and the cells were allowed to incubate for 48 hours. The cells were then lysed and a luciferase reading was taken (Figure 19).
Figure 19. Infection Assay Using Pseudo-Virus Containing Individual CHIKV Envelopes. CHIKV constructs containing individualized structural proteins in pcDNA4 were used to create pseudo-virus as depicted in figure 1. These constructs were used singly and in combination with each other. 48hr virus was harvested and used to infect 293T cells. The data depicts the average of 3 individual trials in 3 independent experiments.

The data would imply that the CHIKV envelope proteins alone or in combination with one another are not enough by themselves to illicit an infection of the pseudo-virus. Due to some
recently published articles verifying the 3D structure or the CHIKV virus and the possible mechanism of proteins processing, it could be speculated that the individualized proteins need to be transcribed together to function properly. With this in mind, a very strong publication would suggest that in order for E2 and E3 to function properly they need to be processed together.

3.3 AIM 2.3 - ANALYZING THE ROLE OF E3/E2 IN PSEUDO-VIRAL INFECTION

The E3 and E2 proteins have recently been suggested to be processed together after infection in the cell. This may explain why no infection was detected using each individual protein separately to illicit an infection in 293T cells. To combat this hurdle a new plasmid was developed through cloning containing the E3 and E2 proteins together in pcDNA 4. This plasmids was used to create a pseudo-virus containing E3 and E2 together. This pseudo-virus was used to infect 293T cells along with a VSV-G positive control, a CHIKV 37997 positive control, and a pcDNA 3.1 positive control.

![Diagram of pcDNA4 Plasmid Containing the E3-E2 CHIKV Combined Envelopes](image)

**Figure 20. Depiction of the pcDNA4 Plasmid Containing the E3-E2 CHIKV Combined Envelopes.** A forward primer for E3 and a reverse primer for E2 were used to amplify CHIKV DNA from pCHIKV37997 in order to
create a strand of DNA containing E3 and E2 together. This PCR amplified strand was ligated into pcDNA4 as performed previously.

With this being said a new construct containing E3 and E2 together was cloned as performed in AIM 2.1 (Figure 20). This new plasmid was transfecting into 293T cells as done above in order to create infectious pseudo-virus. Pseudo-virus was created with the addition of the E3-E2 construct individually or in combination with the E1 plasmid. This infectious E3-E2 pseudo-virus was used to infect 293T cells. The infection was performed as before and luciferase was read in a luminometer (Figure 21).
Figure 21. Pseudo-Viral Infection Assay Performed Using CHIKV Plasmid Containing E3-E2. Pseudo-virus was made using the CHIKV E3-E2 plasmid individually or in combination with CHIKV E1. The pCHIKV37997 and VSV-G were used as positive pseudo-viral controls, and pcDNA3.1 was used as a negative control. The data depicts the average of 3 individual trials in 3 independent experiments.

The data would seem to suggest that even with the E3 and E2 constructs as a single translocational unit, they were not enough to induce a pseudo-viral infection.
3.4 AIM 2.4 - ANALYZING THE CHIKV VIRUS ROLE IN CELL-CELL FUSION

It is known that many viruses cause many cells to fuse into multinucleated cells known as syncytia. In this assay we demonstrate how the expression of CHIKV envelope proteins on the cell surface is sufficient in causing cellular fusion. To accurately measure cellular fusion a Cre-Lox system is used to evaluate cell-cell fusion.

3.4.1 AIM 2.4.1 - CELL FUSION ASSAY

293T cells were plated in 24 well plates. A group of wells were transfected only with the plasmids Stop-Luciferase. This plasmid contains a stop cassette flanked by two lox-p sites followed by a luciferase site. The other wells were transfected with a Cre expression plasmids, along with either pCHIKV 37997, VSV-G, or pcDNA3.1. (A control well transfected with Stop-Luciferase and Cre was also added, along with a Cre only expressing well) (Figure 22). After a 5 hour transfection the media was changed to fresh media and the cells were allowed to incubate for 24 hours. After 24 hours the cells were at approximately 100% confluent. At this point a well containing only Stop-Luciferase was mixed with a well containing a viral envelope and Cre. These mixtures were placed into a microfuge tube and centrifuged to ensure cell mixing. The pelleted cells were then re-suspended in pre-warmed fresh media and added to wells in a 48-well plate. The cells were allowed to incubate for another 24 hours. The cells were then lysed and read in a luminometer.
Figure 22. Cre-LoxP cell-cell fusion expression system. Individual sets of 293T cells were transfected with either Cre + viral envelope, or a stop luciferase construct. If when the cell lines combine there is a compatible receptor the cells will fuse. If fusion is to occur the Cre expressed inside the one cell will remove the loxP sites surrounding the stop cassette, allowing the translation of luciferase.

Figure 23. Close Graphical Analysis of the Cre-Lox System Used in Cell-Cell Fusion. Cyclic Recombinase (Cre) proteins interaction with the LoxP sites causes their asymmetric sequences to interact forming a loop. The DNA loop contains the stop cassette, which typically would stop the production of luciferase, and removes (cleaves) it. The removal of the stop cassette allows for the processing of luciferase, and thus is able to be detected through a luminometer.
When the Cre proteins interact with the lox-p sites surrounding the stop cassette the stop cassette is removed and the luciferase is able to be actively produced (Figure 23). Upon analysis of the data it would appear that the expression of the CHIKV envelope proteins on the 293T cells were sufficient enough to cause cellular fusion as compared to the VSV-G positive control (Figure 24). To specifically pin-point the structural proteins responsible for the cellular fusion individual structural proteins were used.
3.5 AIM 2.5 - ANALYZING INDIVIDUAL CHIKV GLYCOPROTEINS ROLES IN CELL-CELL FUSION

Seeing that the expression of all CHIKV structural proteins caused cellular fusion within 293T cells, we examined the role of the individual expression of CHIKV structural proteins in correlation with cell-cell fusion. The fusion assay was performed in the same manner as before, with the only difference being the use of the individual CHIKV plasmids expressing E3, E2, and E1 individually.
A cell-cell fusion assay was performed as in figure 4 including the individual CHIKV envelope constructs transfected singly or in combination with one another. The data is depicted as fold increase of the Cre infected blank. The data is representative of the average of 3 trials in 3 independent experiments.

As within the pseudo-viral infection assay, it would also appear that the CHIKV plasmids on their own are not sufficient enough to cause cell-cell fusion. A few questions may be raised when looking into this fusion assay. Many times in order to have properly induced cellular fusion a virus needs to have an environment that is fairly acidic. Without also looking at how a
range of pH’s determine the ability of CHIKV to induce cellular fusion, it may be very difficult to speculate upon the results.

3.6 AIM 2.6 - PH DEPENDENT CELL-CELL FUSION USING CHIKV STRUCTURAL GLYCOPROTEINS

A multitude of citric acid buffers were created consisting of various pHs. The pHs were as follows: 7.0, 6.5, 6.0, 5.9, 5.8, 5.7, 5.6, 5.5, 5.4, and 5.0. A cellular fusion assay was performed almost exactly as done previously. The 293T cells were transfected as before and the cells were mixed and distributed to the 48-well plates as done earlier. After the cells were allowed to incubate in the 48-well plates for 24 hours the individual citric acid buffers were added and left to incubate on the cells for 5 minutes. After 5 minutes the buffer was removed and the cells were allowed to recover and incubate for another 24 hours. Unfortunately, after many trials and different buffer time courses most of the cells had died the subsequent day and were unable to be read for fusion. Currently more buffers are being researched to attempt this trial.
4.0 DISCUSSION AND CONCLUSION

Seeing the detrimental results at Reunion Island and the potential for the Chikungunya virus to cause an outbreak in the United States, it appears now more than ever more research is needed to combat this virus. Our current research has shown that the Chikungunya virus was infectious to numerous human cells. Interestingly the virus was shown to infect a blood brain barrier mimic, HBMECs. As to our knowledge there has not been an account previously published showing a correlation to patient symptoms associated with brain infection (such as headache, dizziness, etc). The infection of the blood brain barrier (although not showing an infection of actual brain cells) could provide more information on the possibility of CHIKV in causing infectivity in the brain. A topic also interesting to note is the infection of the A549 alveolar cells. A previous author has suggested that another mechanism of infection could be possible. This was speculated with the high rate of infection seen in the Reunion Island outbreak. They proposed that a possible unknown mechanism of transmission could be possible, such as one of the respiratory tract. Most other data of CHIKV epidemics would most likely show this method as false, but it is very interesting to note the high rate of infection in lung cells seen in the pseudo-viral infection. Humans have been noted as the main reservoirs for the CHIKV virus, although many other animals have been noted to be reservoirs besides humans. The infection of the VERO African green monkey cells would show that other species of mammals are in fact able to be infected besides humans. This would provide more evidence on the basis that monkeys can
be reservoirs for CHIKV. The only human cell line that seemed immune from CHIKV infection that was tested would appear to be T-cells.

After testing the CHIKV pseudo-virus on numerous cell lines, we wanted to analyze the individual CHIKV glycoproteins. The pseudo-viral infections using individualized glycoproteins suggested that the individualized proteins themselves were not sufficient in causing pseudo-viral infection. Looking into recent studies, it would appear the complex processing of the CHIKV proteins (specifically the E3 and E2 proteins) could be causing the lack of infection. Noting this, a new construct was created containing E3 and E2 together. This plasmid also failed to illicit a luciferase response, even with the co-transfection of E1. With the pseudo-virus containing all structural proteins being able to infect the cells, it would appear we are missing a key point with the individualized proteins. It is quite possible that the mechanism of protein processing for CHIKV needs all key proteins together in order to be processed. We examine this further by analyzing the role of CHIKV and its individualized glycoproteins in cell-cell fusion.

The full CHIKV 37997 construct was able to cause cellular fusion in 293T cells. As seen in the pseudo-viral infection assay, using individualized glycoproteins was not sufficient in causing cellular fusion. The complex processing and distribution of the glycoproteins on the cellular surface most likely is the key to the negative results seen in this assay. However, it is also possible there is a pH dependent mechanism negating the fusion process. To ensure pH was not playing a key role in the mechanism of cell-cell fusion, multiple citric acid buffers were used during the fusion assay. The buffers used unfortunately caused a high amount of cell death, and no luciferase readings were able to be read. This experiment is currently being tweaked in order
to limit the amount of cellular death. Looking at the data of the full CHIKV construct being compared to the VSV-G positive control, it would appear the CHIKV is in fact giving a fairly good reading of cellular fusion. This would suggest the data, although not tested to its maximum potential, is fairly reasonable. Individualized CHIKV glycoproteins were also analyzed by cellular fusion.

Each individual CHIKV envelope protein was tested for cellular fusion capabilities. The data would suggest that these glycoproteins individually or in unison with one another do not seem to grant cellular fusion. This data would also coincide with the data received from the pseudo-viral infection assay, showing that without the full construct the individualized plasmids do not seem to show a response. Most probably with the same reasoning in mind as before, the processing and expression of the CHIKV glycoproteins is more complex than placing each individual protein in the cell for expression. It is very probable that the glycoproteins need to be in one continuously read strand to be processed and expressed correctly.

From this data it can be seen that CHIKV can infect a multitude of human cells, but appears to be unable to do so without the expression of all plasmids in a single construct. CHIKV was also able to illicit cellular fusion within 293T cells, but unable to do so when only individualized plasmids were expressed. This data could be very useful in the fact that knowing if one individual envelope protein is disrupted, it could possibly make the virus unviable.

Without a proper vaccine or treatment to the Chikungunya virus, now more than ever we need more research to combat it. The new emergence of mutations causing a switch in mosquito
vector could mean an epidemic in the United States is not out of our near future. The research presented here gives a solid understanding of the cellular tropism of the virus, and the behaviors of individualized glycoproteins in cellular infections and cellular fusion. In order to investigate this issue further, constructs containing the 6K region of CHIKV have been created in order to assess its possible association with viral entry and cellular fusion. The E3/E2 construct is also further being investigated, to examine its role in cellular fusion. This data will hopefully inspire more research, and/or help other scientist contribute to a global eradication of the Chikungunya virus.
5.0 MATERIALS AND METHODS

Transfection

Transfections were performed using either 293T or 293T LentiX cells. 293T were plated in the desired size wells to reach a confluence of approximately 80%. Once the cells reached the desired confluence a transfection media was prepared. Plasmid DNA was added to Optimem solution, along with PEI at a concentration double to the amount of DNA added. The Optimem solution containing DNA and PEI was allowed to incubate at room temperature for approximately 30 minutes. During this incubation time 293T cells had their complete DMEM media replaced with pre-warmed DMEM media containing only 10% FBS. After the 30 minute incubation time and the replacement of the media, the Optimem solution containing plasmid DNA and PEI was added to the 293T cells slowly drop wise. The cells were then placed back into the incubator for 5 hours. After 5 hours the media was then changed from DMEM containing only 10% FBS to complete DMEM media.

Chikungunya Pseudo-virus

Chikungunya pseudo-virus was created by transfecting the CHIKV plasmid synthesized by Genewiz, ∆R8.2 (HIV Gag-Pol), and pTrip Luciferase. These were all transfected at 1:1 ratios. After the transfection was completed the cells were allowed to incubate for 24 hours. When 24 hours was over the DMEM complete media was changed with new complete DMEM media. At
the end of 48 hours the pseudo-virus was harvested. To harvest the virus the cells and media was spun in a centrifuge at 5000 x g for 5 minutes. After 5 minutes the media was removed from the pellet and filtered through a 0.45 µm filter syringe and aliquoted as deemed appropriate and frozen or used immediately for infection. (All controls, such as VSV-G and pcDNA 3.1 blank, were transfected in the same manner).

**Viral Infection**

Once virus had been harvested after 48hrs polybrene was added at a concentration of 4ug/µL. Cell media was removed and the virus containing polybrene was then added to the determined cells. Cells were at a concentration of approximately 70% before they were infected. The virus was left to incubate on the cells in the incubator for 5 hours and was then removed. Fresh pre-warmed complete media was used to replace the virus media.

**Cell-Cell Fusion**

293T cells were plated in 24 well plates. The cells were allowed to grow to approximately 70% confluence. A transfection was then performed on the cells. One batch of cells were transfected only with the Stop-Luc plasmid. The other batch of cells were transfected with viral DNA and a Cre-expression plasmid. The next day when the cells were approximately 100% confluent a well containing the viral DNA and Cre was mixed with a well containing the Stop-Luc plasmid. The mixed wells were placed in a microfuge tube and spun in a centrifuge at 5000xg for 5 minutes. After the cells were centrifuged they were resuspended in fresh DMEM media. The cells were then plated in a 48-well plate to ensure the cells would be 100% confluent the next day. The
cells were allowed to incubate for 48 hours. The cells were then lysed with 1x passive lysis buffer and read on a luminometer after the addition of a luciferase activating reagent.
BIBLIOGRAPHY


