October 2017

Validation of Chimeric Viruses in Plaque Reduction Neutralization Test in Arboviral Disease Diagnostics

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Validation of Chimera Viruses in Plaque Reduction Neutralization Tests for Arboviral Disease Diagnostics

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Public Health
with a concentration in Global Communicable Diseases
Department of Global Health
College of Public Health
University of South Florida

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Date of Approval:
October, 27, 2017

Keywords: Plaque reduction neutralization test, chimera viruses, arboviruses

Dedication

To my Lord and Savior Jesus Christ, for never leaving nor forsaking me and for always providing, comforting, and guiding me in all my ways. To my parents, Elder Keith and Caroline Boykin, who always supported on all my darkest days. To my loving fiancé, who manage to make me forget about all the challenges in my life.

Acknowledgements

It has been such a wonderful experience having been able to conduct research at the Florida Department of Health Bureau of Public Health Laboratories. I would like to thank Mrs. Lea Heberlein-Larson for not only allowing me to conduct research at the lab, but also proposing thesis ideas. I would like to thank the staff at the laboratory that assisted in my research. I would personally like to thank three staff members, Marshall Cone, Lylah Seaton, and Rita Judge for mentorship, for teaching me the PRNT procedures and for helping and teaching me how to make monolayer cell culture plates, respectively. I would also like to thank Dr. Reina, Dr. Unnasch, and Dr. Seyfang for sitting on my committee, advising, and supporting me throughout the process.

With my deepest appreciation and gratitude, I would like to thank Dr. Alexis LaCrue who helped me conduct the research, for guiding and supporting me through the process, for personally taking time out of your schedule to meet me with to rehearse the oral defense, for providing words of wisdom, and for providing emotional support when outside circumstances occurred.

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List of Symbols and Abbreviations

Symbols and Abbreviations	Description
ºC	Degrees Centigrade
%	Percent
α	Alpha
κ	Kappa
ABSA	American Biological Safety Association
APHIS	Animal Plant Health Inspection Services
APHL	Association of Public Health Laboratories
BMBL	Biosafety in Microbiological and Biomedical Laboratories
BPHL	Bureau of Public Health Laboratories
BSA	Bovine Serum Albuminin
BSL-2	Biosafety-level 2
BSL-3	Biosafety-level 3
CDC	Center for Diseases Control and Prevention
CHIKV	Chikungunya virus
CLIA	Clinical Laboratory Improvement Amendment
CMS	Center for Medicare and Medicaid Services
CO_2	Carbon Dioxide Gas
DVBD	Division of Vector-Borne Diseases
DMEM	Dulbecco's Modified Eagle Media
DOH	Department of Health
EEEV	Eastern Equine Encephalitis Virus
ELISA	Enzyme-linked Immunosorbent Assay
EMEM	Essential Minimal Eagle Media

FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FDOH	Florida Department of Health
HAI	Hemagglutination Inhibition Assay
HHS	Health and Human Services
IgG	Immunoglobulin G
IRB	Institutional Review Board
JEV	Japanese Encephalitis virus
LACV	LaCrosse Encephalitis virus
LAI	Laboratory acquired infection
µl	microliter
ml	milliter
NIH	National Institute of Health
NS	Non-structural
ORF	Open Reading Frame
PFU	Plaque forming unit
PHAC	Public Health Agency of Canada
P/N	Positive over Negative value
PAPR	Powered Air Respirator
PBS	Phosphate Buffer Saline
PPE	Personal Protective Equipment
PRMN	Fluorescent-based Plaque Reduction Micro Neutralization Test
PRNT	Plaque Reduction Neutralization Test
SINV	Sindbis virus
SINV/EEEV	Eastern Equine Encephalitis chimera virus
SLEV	St. Louis Encephalitis virus
SVD	Serum Virus Diluent
TCID	Tissue Culture Infectious Dose

TNTC	Too Numerous To Count
USDA	United States Department of Agriculture
USF	University of South Florida
VEEV	Venezuelan Equine Encephalitis virus
WEEV	Western Equine Encephalitis virus
WHO	World Health Organization
WNV	West Nile Virus
YF/SLE	St. Louis Encephalitis chimera virus
YF/WNV	West Nile chimera virus

Abstract

The plaque reduction neutralization test (PRNT) is a confirmatory diagnostic assay that is used to confirm a variety of diseases. The performance of PRNT requires the use of infectious wild type viruses, which increases the risk of laboratory acquired infections. For instance, eastern equine encephalitis (EEEV) is a highly virulent pathogen used in PRNT that can result in potentially fatal neurological diseases among humans and equines. Therefore, arboviral PRNT must be performed in Biosafety Level 3 (BSL-3) containment facilities and may require select agent approved scientists, like in the case of EEEV. These stringent requirements restrict the ability of public health laboratories to conduct PRNTs. Chimera viruses, recombinant constructs that have been bio-engineered to express the immunogenic structural proteins from the wild type virus in an attenuated form, can serve as a substitution for infectious viruses when performing PRNT. Since chimera viruses do not require the use of a BSL-3 facility and are not classified as select agents, their use offers advantages over wild type viruses. This study aimed at validating the use of EEE and West Nile chimera viruses as an alternative to the corresponding wild type viruses for diagnostic purposes at the Florida Department of Health (FDOH) Bureau of Public Health Laboratories (BPHL). These evaluations were conducted using human and avian sera. The results illustrate that chimera virus-based PRNT portrays specificity comparable to that of the wild type virus, while a slight reduction in sensitivity was observed when human sera was used. Considering their benefits in increasing safety and reducing regulatory requirements, these chimera viruses are an important alternative to the virulent wild type viruses and could be highly beneficial for diagnostic laboratories.

Chapter One: Introduction

Arboviruses: Overview

Arboviruses are zoonotic viruses that are transmitted from an arthropod vector to a vertebrate host (Go, Balasuriya, & Lee, 2014; Weaver & Reisen, 2010). Over 100 species of arboviruses have been identified and they are transmitted to humans and other mammals by infected, hematophagous female arthropod vectors, such as mosquitoes and ticks (LaBeaud, Bashir, & King, 2011; Liang, Gao, & Gould, 2015; Weaver & Reisen, 2010).

Currently, arboviral diseases represent over 30% of all emerging infectious diseases. This global representation is due to factors such as climate change, population increase, and urbanization (Jones et al., 2008; Hollidge, Weiss, & Soldan, 2011; Liang, Gao, & Gould, 2015). In regards to climate change, most vectors thrive in high climate temperatures (i.e. 80^{o}F and above); therefore, the majority of arboviruses that are associated with human and animal diseases circulate within tropical and subtropical regions of the world (Liang, Gao, & Gould, 2015). For example, countries in Southeast Asia (i.e. Japan and India), North, South, and Central America (i.e. U.S., Brazil, and Costa Rica), Sub-Saharan Africa, the Caribbean, and the Mediterranean have a high prevalence of arboviral infections transmitted by *Culex spp* and *Aedes spp* mosquitoes (Gould & Solomon, 2008; Liang, Gao, & Gould, 2015; Patterson, Sammon, & Garg, 2016). In Florida, the hot temperature climate and long rainy seasons provide optimal breeding conditions for mosquitoes (Adalja, Sell, Bouri, & Franco, 2012; Gargano et al., 2013). Nevertheless, the geographical range in which arboviruses circulate has been and continues to be extending beyond tropical and subtropical regions of the world (Go, Balasuriya, & Lee, 2014).

Urbanization and increasing population have caused humans to encroach on mosquito habitations which may increase the risk of transmission of arboviral infections (Go, Balasuriya, & Lee, 2014; Gould & Solomon, 2008; Liang, Gao, & Gould, 2015).The increase in the incidence of arboviral diseases may lead to increased cost associated with diagnosis, surveillance, and treatment of arboviruses which may translate into a greater economic burden (Barrett, 2014; Liang, Gao, & Gould, 2015). For example, since 1999, hospitalizations caused by infections with West Nile Virus (WNV) cost approximately $800 million dollars in the U.S. (Barrett, 2014).

Arboviruses: Classifications

Arboviruses consist of a large group of zoonotic viruses that infect arthropods. These viruses are typically classified into the *Togaviridae* family, *Flaviviridae* family, *Bunyaviridae* family, and *Reoviridae* family (Atkins, 2013; Beckham & Tyler, 2016; Hollidge, Weiss, & Soldan, 2011). For the interest of this study, only the *Togaviridae* and *Flaviviridae* family will be described.

The *Togaviridae* has a genome that ranges from 7 to 11.8 kb in length, is non-segmented, and contains positive sense-single stranded RNA (Garmashova et al., 2007). This family consists of the *Rubivirus* and *Alphavirus* genera, only the latter contains arboviruses. Alphaviruses are small enveloped RNA viruses that contain two open reading frames (ORF) within their genome (Atkins, 2013; Hollidge, Gonzalez-Scarano, & Soldan, 2010; Martinez, Snapp, Perumal, Macaluso, & Kielian, 2014). The *Alphavirus* genus contains 26 arboviruses (Powers et al, 2001). There are 29 known alphaviruses that are transmitted worldwide that include, but are not limited to, eastern equine encephalitis virus (EEEV), western equine encephalitis virus (WEEV),

venezuelan equine enecphalitis virus (VEEV), Sindbis virus (SINV), and chikungunya virus (CHIKV, Hollidge, Gonzalez-Scarano, & Soldan, 2010).

The *Flaviviridae* family consists of the *Flavivirus* genus. This genus contains over 75 identified arboviruses of which approximately 40% are mosquito-borne, while 16 are tick-borne, and the remaining 18 have no known vectors (Heinz et al., 2000; Hollidge, Gonzalez-Scarano, & Soldan, 2010;). Flaviviruses are positive sense single stranded enveloped RNA viruses that are spherical in shape (Yu et al., 2008). Their non-segmented genome generates ten mature viral proteins by the proteolytic processing of a single polyprotein (Stadler et al., 1997). The viral proteins include three structural proteins that consist of a capsid, envelope, premembrane, and seven nonstructural proteins. This genus contains more than five of the most well-studied encephalitis-causing arboviruses which include WNV, St. Louis encephalitis virus (SLEV), La Crosse encephalitis virus (LACV), and Japanese encephalitis virus (JEV, Atkins, 2013; Hollidge, Gonzalez-Scarano, & Soldan, 2010).

Arbovirus: Prevention

Preventative control measures are the best method of protection against arboviral infections. Suggested mosquito control measures include: wearing protective clothing such as long pants and long sleeved-shirts, using bed nets, avoiding outside activities during hours when mosquitos are most active, staying in air-conditioned environments, and using mosquito repellants (Center for Disease Control and Prevention, 2016; Florida Department of Health, 2017).

Alphavirus: Eastern Equine Encephalitis

Overview. Eastern equine encephalitis virus (EEEV) was first discovered in 1831 when 75 horses mysteriously died of viral encephalitis in Massachusetts (Morris, Callahan, & Howard, 1988). In humans, EEE has a morbidity rate of approximately 90% and a mortality rate of 30-75%, especially in those over the age of 50 and under the age of 15 (Armstrong & Andreadis, 2013; Zacks & Paessler, 2010). There have been a total of 270 human cases of EEE reported from 1964 to 2010 (Hollidge, Gonzalez-Scarano, & Soldan, 2010).

The genome of EEEV is approximately 11.7kb in length, which includes a 5'cap and a 3' poly (A) tail. The 5' end of the genome encodes for four nonstructural proteins, NSP1 to NSP4 (Young et a., 2008). The 3' end of the genome encodes a sub-genomic RNA (26S) that produces three main structural proteins which include a capsid and envelope glycoproteins E1 and E2 (Arrigo, Adams, & Weaver, 2010; Weaver et al., 1994).

Originally, the virus was divided into two strains, North and South American, based on their antigenic properties. However, additional antigenic and phylogenetic analyses have reclassified the virus to include four subtypes that correspond to four major genetic lineages, named lineages I to IV (Arrigo, Adams, & Weaver, 2010; Atkins, 2013). The North American EEEV strains and several of the Caribbean strains consist of lineage I, whereas the South and Central American EEEV strains comprise lineages II to IV (Beckham & Tyler, 2016; Brault et al., 1999). Lineages II to IV have become a separate viral species known as the Madariaga virus (Weaver et al., 1994).

Life Cycle. Transmission of EEEV occurs when an infected female mosquito takes a blood meal from an avian host and transmits EEEV (**Figure 1**). *Culiseta melanura and Culiseta morsitans* are the predominant vectors that transmit EEEV to the avian host (Atkins, 2013; Morris, Callahan, & Howard, 1988; Vander Kelen, Downs, Stark, Loraamm, Anderson, &

Unnasch, 2012; Zacks & Paessler, 2010). Other mosquito species such as *Aedes vexans*, *Coquillettidia petrubans, Ochlerotatus Canadensis,* and *Oc. sollicitans,* among others, have been implicated as epizootic bridge vectors from viremic birds to equines, domesticated animals, and humans (Armstrong & Andreadis, 2008, 2010; Cupp, Klinger, Hassan, Viguers, & Unnasch, 2003; Morris, Callahan, & Howard, 1988; Vander Kelen, Downs, Stark, Loraamm, Anderson, & Unnasch, 2012). Eastern equine encephalitis virus is known to replicate at the site of infection, typically in non-neural tissues such as the lymphatic system or tissue adjacent to the mosquito bite (Arrigo, Adams, & Weaver, 2010; Jose, Snyder, & Kuhn, 2009). Post-transmission to a mammalian host, EEEV enters through the host subcutaneous and cutaneous tissues through cellular receptors (Strauss, Rumenapf, Weir, Kuhn, Wang, & Strauss, 1994). The virus, then, migrates to the lymph nodes where it reaches the bloodstream. In the bloodstream, EEEV binds to specific tissue receptors, undergoes endocytosis and initiates an RNA-dependent synthesis as well as protein synthesis (Jose, Synder, Kuhn, 2009; Strauss, Rumenapf, Weir, Kuhn, Wang, & Strauss, 1994).

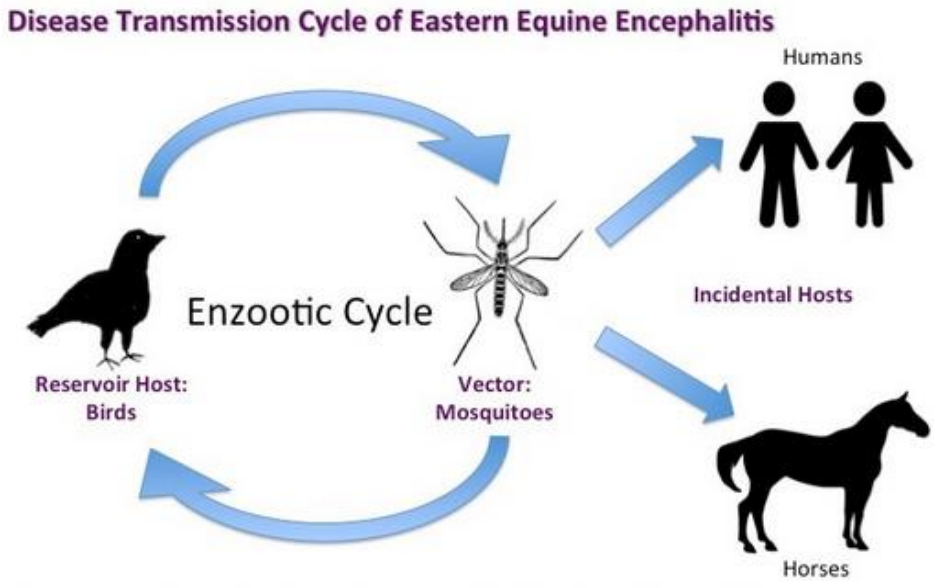

Figure 1. This figure shows the transmission cycle of Eastern Equine Encephalitis virus. Source: Cornell University, College of Veterinary Medicine (2017, November). Eastern Equine Encephalitis.

Symptoms. Infection with EEEV can either be systemic or encephalitic (Armstrong & Andreadis, 2013). Occasionally, those who become infected with EEEV may be asymptomatic (Deresiewicz, Thaler, Hsu, & Zamani, 1997). The incubation period for EEEV is 4 to 10 days in humans and 5 to 14 days in equines (Armstrong & Andreadis, 2013; Zacks & Paessler, 2010). Clinical manifestations of a systemic infection typically occur abruptly and include fevers, abdominal pain, chills, arthralgia, weakness, headache, and myalgia (Deresiewicz, Thaler, Hsu, & Zamani, 1997; Heymann, 2015). The prodrome has a duration lasting one to two weeks and recovery is complete when there is no sign of central nervous system involvement (CDC, 2016a). An estimated 35% of those who survive are left with disabling and progressive mental and physical sequelae, with neurological symptoms ranging from minimal brain dysfunction to

severe intellectual dysfunction (Vander Kelen, Downs, Stark, Loraamm, Anderson, & Unnasch, 2012).

Diagnosis and Treatment. Diagnosing EEEV infection can be difficult due to the similarities that are shared among other encephalitic infections such as LaCrosse virus, tuberculosis, coxsackieviruses, naegleria infection, legionnaires disease, and rabies (Gaensbauer, Lindsey, Messacar, Staples, & Fischer, 2014). Unfortunately, there is no readily available cure or vaccine for humans against EEEV. Treatment consists only of supportive care (Gaensbauer, Lindsey, Messacar, Staples, & Fischer, 2014; Heymann, 2015). However, there are three vaccines available for equines (United States Department of Agriculture, Animal Plant Health Inspection Service, 2008). The vaccines are monovalent, bivalent, and trivalent. Owners of horses are recommended to scrutinize which vaccine is best to use depending on the local prevalence of the disease (FDOH, 2017; USDA, APHIS, 2008).

Future Research. EEEV will continue to remain a public health concern. Currently, researchers are working on human vaccines for EEEV infection. Investigation EEEV vaccines and for other alphaviruses (i.e. Venezuelan equine encephalitis) may become available for people who are at high risk for infection; however availability will be limited (FDOH, 2017; Heymann, 2015).

Flavivirus: West Nile Virus

Overview. West Nile virus (WNV) is one of the earliest recognized arthropod-borne viral diseases of man (Gubler, 2007). It was first isolated in 1937 in the homonymous province of Uganda from the blood of a febrile woman (Briese et al., 1999; Gubler, 2007; Kramer, Li, & Shi, 2007). Between 1999 and 2014 there have been 41,762 human cases (inclusive to 18, 810

neruoinvasive cases) of WN disease that have been reported to the CDC (National Institute of Health, 2015). In 2014, there were 2,205 cases and in 2015 there were 2,060 cases (Donadieu et al., 2013).

The genome of WNV is approximately 11kb in length and encodes for three structural proteins (capsid, pre-membrane, and envelope) and seven nonstructural proteins (Castle, Nowak, & Wengler, 1985). The capsid protein is associated with the genome to form the nucleocapsid which is also enclosed by a lipid bilayer with E proteins protruding outward (Castle, Nowak, Wengler, & Wengler, 1986). The E proteins facilitate cellular attachment and membrane fusion and are a significant virulence factor (Marfin & Gubler, 2001).

There are two genetic lineages of WNV; Lineage 1 consists of isolated strains from Europe, Middle East, South Asia, North America, some African strains, and Australia (Chancey, Grinev, Volkova, & Rios, 2014). This lineage entails the strains that have caused encephalitic outbreaks among humans and horses (Jia et al., 1999). Lineage 2 is comprised of southern Africa strains, central Africa, and the original Ugandan strain isolated in 1937 (Komar, 2003). Theses genetic lineages continually occur and are maintained in nature by a WNV primary host (Paz, 2015).

Life Cycle. West Nile virus is typically transmitted by *Culex spp* mosquitoes. However, it can also be transmitted via *Aedes* and *Anopheles* (Hayes et al., 2005). *Culex tarsailis* is the primary mosquito vector of WNV in the western regions of the United States (Colpitts, Conway, Montgomery, and Fikrig, 2012). West Nile virus is maintained in animal hosts; preferably birds in the Corvidae family i.e. blue jays and crows by mosquitoes (**Figure 2**, Hayes et al., 2005). Equines and humans can acquire WNV as dead-end or incidental hosts (Chancey, Grinev, Volkova, & Rios, 2014). West Nile virus is known to replicate at the site of infection. After a

mosquito takes a blood meal from an infected human host, the virus enters through the skin cells through cellular receptors (Gyure, 2009; Lim, Koraka, Osterhaus, & Martina, 2011). This enables the virus to migrate to the lymph nodes and then into the bloodstream (Cho & Diamond, 2012; Klein & Diamond, 2008). Viral entry of the central nervous system follows activation of toll-like receptors and tumor necrosis factor-α which increases the permeability of the virus crossing the blood-brain barrier (Cho & Diamond, 2012; Klein & Diamond, 2008; Samuel & Diamond, 2006). West Nile virus directly infects neurons within the deep nuclei and gray matter area of the brain, brainstem, and spinal cord (Hayes, Sejavrt, Lanicotti, Bode, & Campbell 2005; Sitati & Diamond, 2006).

Symptoms. Approximately 80% of human infections with WNV are asymptomatic. Symptoms can range from flu-like malaise to severe neuroinvasive disease (Paz, 2015). Less than 1% of human infections progress to severe WN disease (Chancey, Grinev, Volkova & Rios, 2014). Seroepidemiological studies have demonstrated that 20-25% of WNV infected individuals develop mild illness and about 0.67% develop WN neurological disease (Beckham & Tyler, 2016; Chancey, Grinev, Volkova & Rios, 2014; Hayes et al., 2005). Neurological cases frequently consist of meningoencephalitis (Komar, 2003). Encephalitis is only one of several severe symptoms caused by WNV. The incubation period for WNV in humans is between 2 and 14 days. (Hayes et al., 2005; Petersen & Marfin, 2002). Though infections are subclinical and often asymptomatic, occasionally clinical manifestations develop 2 to 21 days post infection (Hayes et al., 2005; Petersen & Roehrig; 2001). Cases where neurological symptoms do not develop and patients are not hospitalized are referred to as WN fever (Komar, 2003).

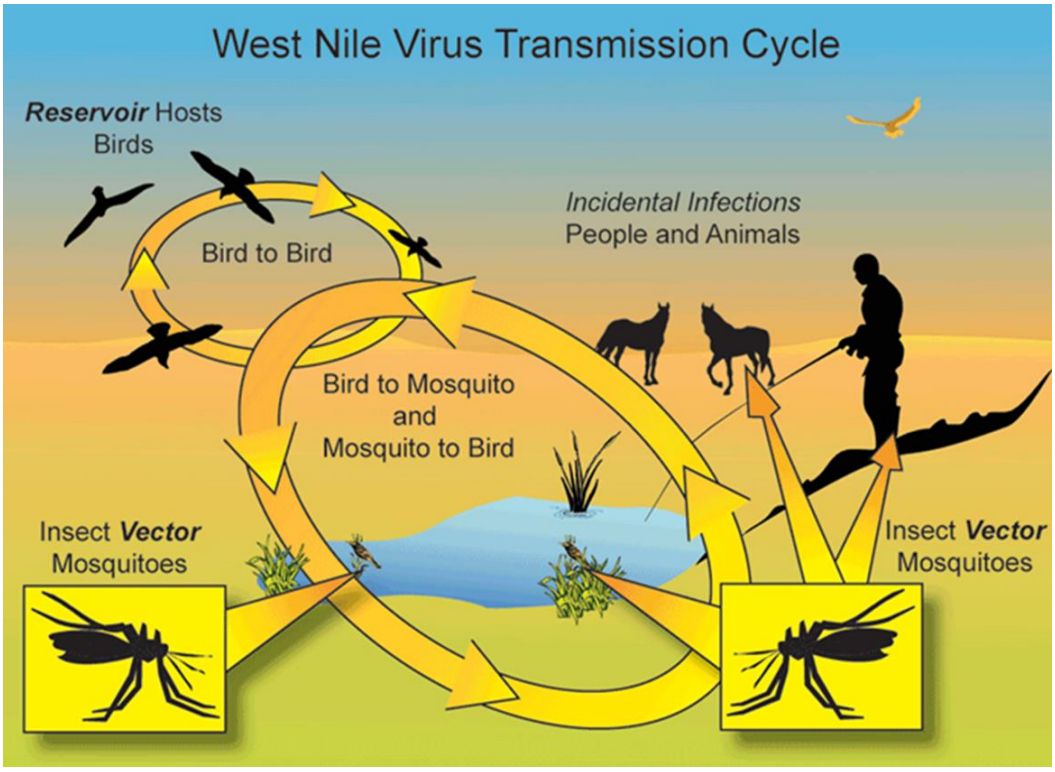

Figure 2. This figure shows the transmission cycle of West Nile virus. Source: Paxtang Borough:Harrisburg, PA. (2017, November).

Diagnosis and Treatment. Diagnosis of WNV infection can be challenging due to the clinical similarities the virus shares with other infectious agents such as EEEV and SLEV (Hollidge, Gonzalez-Scarano, & Soldan, 2010). WNV-related meningitis and encephalitis are clinically indistinguishable from meningitis and encephalitis related to other viral diseases. Movement disorders such as Parkinson's disease and poliomyelitis share similar pathogenesis with WNV encephalitis (Komar, 2003). West Nile virus can also be associated with Guillain-Barre syndrome and radiculopathy (Gyure, 2009; Petersen & Marfin, 2002).

Unfortunately, there is no vaccine and there are no specific antiviral treatments for WNV infections (NIH, 2015). Over-the-counter pain relievers can be used to mitigate WN disease symptoms (Chancey, Grinev, Volkova & Rios, 2014; Komar, 2003). For cases that require

hospitalization, supportive treatment such as intravenous fluids can be administered (CDC, 2016b).

Future Research. Research focusing on vector control strategies such as reducing the abundance of mosquitoes, developing more effective repellent, and understanding mosquito behavior needs to continue (Hayes et al., 2005; Petersen & Roehrig, 2001). In addition, the development of a vaccine for WNV is highly needed. There is a NIH-funded trial for a vaccine that is known as HydroVax-001 (NIH, 2015). HydroVax-001 is a hydrogen peroxide-based technique that causes the WNV to be inactive while maintaining the triggering surface structure of the immune-system (NIH, 2015).

Chapter Two: Laboratory Diagnostic Techniques

Neutralization

Plaque Assays. Plaque assays are one of the most widely practiced techniques that are used to measure the ability of multiple infectious virions to form a plaque (i.e. a group of infected cell) on a confluent monolayer of cultured cells (Shurtleff et al., 2012). These assays are used for virus isolation, purification of viral clonal populations, and determining the titer of viruses (Baer & Kehn-Hall, 2014; Juarez, Long, Aguilar, Kochel, & Halsey, 2013).

Plaque Reduction Neutralization Test. Plaque reduction neutralization test (PRNT) is a commonly used type of plaque assay that is implemented worldwide for diagnostic purposes. This test is considered to be the gold standard for detecting and measuring antibodies that can neutralize viruses because it has a higher sensitivity than other tests (i.e. hemagglutination inhibition and enzyme-linked immunosorbent assay) without compromising specificity (Baer & Kehn-Hall, 2014; Gennaro, Lorusso, Casaccia, Conte, Monaco, & Savini, 2014). A fixed amount of viral suspension is added to serum dilutions of each sample (Juarez, Long, Aguilar, Kochel, & Halsey, 2013). The viral-serum mixture is then incubated to allow the antibodies, if present in the subject sample, to attach to and neutralize the virus (Dulbecco & Vogt, 1953). The mixture is then added to a monolayer of confluent cells (Shurtleff, Keuhne, Biggins, & Keeney, 2011). An agarose mixture is placed onto the cells to limit the virus from spreading indiscriminately (Klebe & Harriss, 1984). The cells are incubated for one to four days to allow for viral growth and the formation of plaques. The plaques are counted using a vital dye such as neutral red (Shurtleff et al., 2012). Viral titers are measured in plaque forming units (PFU) per milliliter and are calculated by taking the reciprocal of the highest serum dilution that can reduce the number of

plaques by 90% compared to the serum free virus (Baer & Kehn-Hall, 2014; BD Biosciences, 2017; CDC, 2016c; Martrosovich, Martosovich, Garten, & Klenk, 2006; Mckeating, 1991).

Multiple virions could potentially infect a single cell, therefore the term plaque forming unit (PFU) is used (CDC/DVBD, 2017; Martrosovich, Martosovich, Garten, & Klenk, 2006). Plaque morphology such as plaque size, border definition, clarity, and distribution provide valuable information concerning the growth and virulence factors of the virus (Abedon & Yin, 2009). However, the morphology of the plaques are heavily impacted by the growth conditions of the cells (Abedon & Yin, 2009; Baer & Kehn-Hall, 2014).

Use of Wild Type Viruses. Performance of arboviral PRNT requires the use of a Biosafety Level 3 (BSL-3) containment facility when working with risk group 3 biological agents such as EEEV and WNV are used (Komar, Langevin, & Monath, 2009). These agents possess the capability of causing severe human disease (i.e. encephalitis), therefore, if exposed laboratory personnel are at an increased for acquiring an infection because there is no curative treatment available (American Biological Safety Association, 1988; Coelho & García Díez, 2015; Johnson et al., 2011; Public Health Agency of Canada, 2009; Tun, Sadler, & Tam, 2006; World Health Organization, 2003).

Training. Manipulation of risk group 3 agents within a BSL-3 facility requires extensive training and clearances, several layers of personal protective equipment (PPE), and various background checks (ABSA, 1988; Johnson et al., 2011; Tun, Sadler, & Tam, 2006). Furthermore, use of select agents (i.e. EEEV) requires laboratorians to be select agent approved by the Federal Select Agent Program, under the CDC. According to the Select Agent Program, each laboratory must have a limited number of select agent approved personnel that can be trained (Bureau of Public Health Laboratories, 2015). Additionally, there are a finite number of

public health labs that are equipped with a BSL-3, much less enrolled in a Select Agent program (Johnson et al., 2011).

According to the CDC Biosafety in Microbiological and Biomedical Laboratories (BMBL, 5th Ed., 2009), a BSL-3 facility is used to perform diagnostic, clinical, teaching, or research with exotic or indigenous agents that may cause a serious or potentially lethal disease through the transmission route of inhalation due to exposure. All laboratory personnel are required to receive specialized training to handle such pathogens (ABSA, 1988; Tun, Sadler, & Tam, 2006). The laboratorians must be supervised by a scientist who is familiar with the associated procedures and is competent in handling infectious agents (Fleming, 1995; WHO, 2004). All procedures that incorporate the manipulation of potentially infectious materials must be conducted within a class II or class III biosafety cabinet (WHO, 2004).

At the Tampa Bureau of Public Health Laboratory (BPHL), all personnel must wear PPE that is in compliance with the BMBL. Specifically, there are two types of PPE that a laboratorian can choose to wear (ABSA, 1988; Tun, Sadler, & Tam, 2006; WHO, 2004). One consists of using an N-95 respirator and the other entails the usage of a powered air respirator (PAPR). Laboratorians that choose to work with the N-95 respirators are subject to fatigue, physical and mental exhaustion, and a reduction in efficiency (Tun, Sadler, & Tam, 2006). However, with the N-95 respirator, peripheral vision is not distorted and flexibility and adequate range in motion is allowed (Fleming, 1995; WHO, 2003). Whereas the PAPR, reduces the peripheral vision of the users, thus creating opportunities for error, spills, and other mistakes to occur (WHO, 2004). However, the PAPR reduces the facial stress that the N-95 causes (Tun, Sadler, & Tam, 2006). Due to these conditions, it is highly recommended that laboratorians use the buddy system and

not work alone (BPHL, 2015). Thus, these conditions create an ineffective and unsafe work environment for laboratorians (Tun, Sadler, & Tam, 2006).

Laboratory acquired infections. In spite of all of the necessary precautions, protocols, and procedures that are in place, accidents and laboratory acquired infections (LAIs) can still occur (Fleming, 1995; Pike 1976; Sewel, 2006;). Approximately, 45% of clinical diagnostic laboratories account for the total number of LAIs (CDC, 2009; Sewell, 1995). Overall, there has been an increase of cases that have been reported regarding laboratory acquired infections, most of which involve microbiological agents belonging to risk group 3 (Fleming, 1995; Pike, 1979; Sewell, 2006; WHO, 2004).

According to Nagata, Wong, Wu & Hu (2013), four laboratory acquired-cases of EEEV and sixteen cases of WEEV were documented, with four of the sixteen cases resulting in fatality. Primary hazards that can result in an accidental EEEV inoculation include viral contact with broken skin or mucous membranes, bites from infected mosquitoes or rodents within the laboratory (Fleming 1995, Pike, 1979; PHAC, 2010; Sewel, 1995). Another arbovirus that has caused several LAIs is WNV. In 2002, the CDC reported two cases of WNV infection in laboratory workers, both were microbiologists, without other known risk factors who acquired the infection through percutaneous inoculation. Since then, over 20 West Nile virus- laboratory acquired infections have occurred (CDC, 2002).

Capacity. Select agents, such as wild type EEEV, require classified Health and Human Services (HHS) and USDA select agent BSL-3 containment facilities (Johnson, Kosoy, Hunsperger, Beltran, Delorey, Guirakhoo, & Monath, 2009). This prevents public health laboratories that are only equipped with BSL-2 facilities and laboratories that have non-select

agent approved personnel from confirming arboviral infections via PRNT (Johnson et al., 2011; Komar, Langevin, & Monath, 2009).

Turnaround Time. Typically, PRNT takes one to two weeks to complete; however, this varies depending on the virus that is being confirmed. Wild type viruses, especially flaviviruses, grow very slowly and tend to produce small sized plaques (Gennaro, Lorusso, Casaccia, Conte, Monaco, & Savini, 2014). Due to length of time required to complete a PRNT assay, turnaround times for diagnostic purposes are increased (BPHL, 2015). The increased turnaround time imposes a critical, and in some cases detrimental, dilemma for those who are waiting on the diagnostic results in order to make an important medical decision (Rabe et al., 2016). This factor had a significant impact on the pregnant women who were waiting on confirmatory PRNT results for Zika virus to determine if their pregnancy should be terminated or not (Bingham et al., 2016; Rabe et al., 2016).

Chimera Viruses. As explained earlier, performance of PRNT using pathogenic wild type viruses places laboratory personnel at an increased risk for laboratory acquired infections, increases turnaround time, and decreases the capacity for public health laboratories to confirm arboviral diagnosis via PRNT (Johnson et al., 2011). Chimera viruses are a safer alternative because they decrease the risk of obtaining an infection, decrease turnaround time, and increase capacity for confirmatory diagnostics within public health laboratories (Johnson et al., 2011; Komar, Langevin, & Monath, 2009; Monath, 2011). Moreover, chimera viruses have demonstrated to produce phenotypically larger plaque sizes that are more definitive to see and count which provide a more accurate titer reading (Johnson et al., 2009).

Chimera viruses are recombinant viruses consisting of a mixture of genes from two or more different viruses constructed from cDNA clones that encode a complete RNA viral genome

(CDC, 2009). Chimeric viruses are composed of the genes and gene products responsible for intracellular replication belonging to the vaccine or attenuated virus and the genes and gene products of the envelope of the virus of interest (Monath, 2001). Since the viral envelope contains all of the antigenic determinants responsible for inducing neutralizing antibodies, the result of infection with the chimeric virus is that such antibodies are generated only against the second virus and will not produce an immunogenic response (Monath, 2001). Chimera viruses possess the equivalent antigenic makeup that will provide comparable sensitivity and specificity to the wild type viruses in the assay (Komar, Langevin, & Monath, 2009). Therefore, chimera viruses should have the capability of being neutralized by antibodies that are elicited during infections of the wild type virus (Johnson et al., 2011).

There are several full-length viruses or truncated replicons that have been bio-engineered from numerous flaviviruses and alphaviruses to produce chimeras. Chimera flaviviruses are constructed from an attenuated yellow fever vaccine virus backbone that is combined with structural protein genes replaced with heterologous virus (Monath, 2001, 2011). Chimera alphaviruses are constructed from a Sindbis virus (a relatively non-pathogenic human alpha virus) backbone that is combined with structural protein genes replaced with heterologous virus (Johnson et al., 2011).

These safe alternatives may be used under BSL-2 containment facilities and practices (Komar, Langevin, & Monath, 2009). Validation of chimera viruses can enhance the capacity in which public health laboratories with limited facilities and Select-Agent approved personnel can perform confirmatory arbovirus diagnostic testing (Johnson et al., 2011). Additionally, use of chimera viruses can also decrease the turnaround times of the patient results because chimeras tend to grow faster than their wild type counterparts (Johnson et al., 2009, Johnson et al., 2011;

Monath, 2011). Plaque reduction neutralization test using chimera viruses allow results to be analyzed within one to two days post inoculation, which drastically reduces the turnaround time from 3-14 days to 2-7 days (Gennaro, Lorusso, Casaccia, Conte, Monaco, & Savini, 2014). A reduction in turnaround times creates the opportunity for timely medical decision to occur and recommendations for prevention, such as mosquito control for the circulating viruses (Johnson et al., 2011). Moreover, chimera viruses have demonstrated to produce phenotypically larger plaque sizes that are more definitive to see and count which provide a more accurate titer reading (Johnson et al., 2009). Although, the chimera virus will not elicit a replicable response as the wild type, the alternative responses are comparably sufficient to determine the serostatus in serological diagnostic testing (Johnson et al., 2011). Overall, chimera viruses are an important and beneficial asset for the purpose of laboratory diagnostics. Their usage in PRNT diagnostics is invaluable.

Rationale of the Study

Therefore, the purpose of this study was to evaluate EEE and WN chimera viruses in the PRNT assay. These chimera viruses are a safer alternative to the wild type pathogen counterparts because they are non-pathogenic to humans while still maintaining antigenicity. Additionally, chimera viruses enhance the capacity of public health laboratories to perform arboviral confirmation tests such as PRNT.

The specific aims of this study were to: 1) assess the serostatus outcomes, sensitivity, and specificity of each chimeric virus and 2) to assess the titer agreement of each chimeric viruses used at the Bureau of Public Health Laboratory (Tampa). Successful completion of this study provides an assessment on the performance of chimeric viruses using different specimen sources

and provides another perspective on performing PRNT overall. Currently, all EEEV and human clinical WNV PRNT testing was performed using wild type viruses for the state of Florida is performed at the Bureau of Public Health Laboratories.

Chapter Three: Materials and Methods

Media. The following reagents were used to make the media and diluent. Bovine Serum Albuminin (BSA) was purchased from Millipore®, Fetal Calf Serum (FCS) was purchased from Hyclone Fischer®, Dulbecco's Modified Eagle's Medium (DMEM), , Hepes, glutamine (essential amino acids), fungizone (antifungal), kanamycin (antibiotic) , penicillin (antibiotic), and streptomycin (antibiotic) were all purchased from Gibco®. The following reagents were used to make the media-agar solution for the first and second overlays in PRNT. Sea Kem Agarose, Sodium Bicarbonate powder, and Neutral red stock solution were purchased from Fischer®.

Viruses. The arboviruses that were used in this study consisted of WNV and EEEV. Eastern Equine Encephalitis Virus strain D64-837 Vero 134 was derived from a patient specimen and was cultured and grown at BPHL-Tampa. SINV/EEEV (North American strain) chimera virus strain number 796 was obtained from the CDC that initially obtained the chimera from the University of Texas Medical Branch, Galveston, Texas (Goodman et al., 2015). West Nile virus strain NY99 Vero 136 was derived from a patient specimen and was cultured and grown at BPHL-Tampa. The Yellow Fever/West Nile ChimeriVax® was obtained from the CDC who initially obtained the chimera from Sanofi Pasteur.

Cells. Vero cells were initially obtained from ATCC® CCL-81™; however, they were then provided by the CDC. Vero cell are culturally maintained at BPHL-Tampa.

Viral Titration. A virus titration was performed for each virus in order to determine the Tissue Culture Infectious Dose, $TCID_{50}$/100 µl. All viral titrations were performed in BD Falcon Tissue Culture © 6-well plates using Vero cells. Vero cells were approximately 90% confluent prior to inoculation. A series of 14 serial virus dilutions were made using 100 ml of serum virus diluent

(SVD) with 8% FCS. The first four viral dilutions were made at a 1:10 dilution, while the remaining nine were half log dilutions. A total of 150 µl of each viral dilution were placed into a 96-well BD Falcon Tissue Culture © plate on top of a coated layer of 150 µl of SVD. The 96-well plate was placed into a humidified chamber and incubated overnight at 4°C. After incubation, media from the 6-well plates was aspirated until approximately 100 µl remained in each well. Following the aspiration, 100 µl of the virus/diluent mixture was added in duplicate. The plates were incubated at 37°C for 1 hour in a 5% CO_2 incubator and rocked every 15 minutes. An overlay of media containing 0.5 ml of each antibiotic, 200mM glutamine, 1x Eagle's Minimum Essential Media (EMEM), 8% FCS, 1% hepes, 8.8% sodium bicarbonate, and 0.5% agar was placed onto the Vero plates, allowed to harden, and placed into a 37°C incubator. After two and four day incubation for EEEV and WNV, respectively, a second overlay media containing 2% of neutral red was placed onto the plates. The plaques were counted on days one and two post second overlay. The dilution containing plaques ranging from 30-100 PFU was chosen as the challenge viral dilution (CDC/DVBD, 2017).

Cell Culture. Vero cells were seeded to be confluent on the day the plates were used for PRNT. BD Falcon Tissue Culture © 6-well plates were used for avian sera samples and 12-well plates were used for human sera samples. The seeding densities ranged from 7.5 to 10 x 10^4. The cells were seeded within this range to ensure that cells were at minimum 90% confluent within 72 hours after seeding (CDC/DVBD, 2017). Cells were incubated at 5% CO_2 and 37°C. The cells were maintained in DMEM with 8% FCS, 1% sodium pyruvate, 3% sodium bicarbonate, and 0.5 ml of antibiotics at 37°C in a 5% CO_2 incubator.

Serum Specimens. Avian and human sera were obtained from the FDOH BPHL-Tampa. Avian sera samples that were tested for EEEV antibodies were obtained weekly from the Florida

Sentinel Chicken Program from January 2016 to December 2016. All samples were initially screened for EEEV and SLEV antibodies using the hemagglutination inhibition assay (HAI). Samples with a titer of 10 or greater in HAI were then screened using enzyme-linked immunosorbent assay (ELISA). For EEEV, the avian samples were selected based on their Positive over Negative (P/N) value in ELISA and on their available volume. Specimens with a (P/N) value greater than or equal to 5.00 and a volume of at least 300 µl were selected for PRNT.

Thirty samples (20 positive and 10 negative) were selected for this study because the Center for Medicare & Medicaid Services (CMS) agency through Clinical Laboratory Improvement Amendent (CLIA) recommends that a minimum of 20 samples should be used to validate an assay (Association of Public Health Laboratories, 2013). Additionally, a sample size of 30 improved the statistical power for this study. Previous PRNT results were blinded to the technician performing PRNT prior to the beginning of the study.

Human sera samples that were tested for EEEV and WNV antibodies were obtained from different Florida County Health Departments or hospitals and were submitted for diagnostic arbovirus testing. Human sera samples were selected based on their original strength in IgG ELISA for EEEV and WNV antibodies. Volume and P/N value selection criteria were the same as the ones for avian samples. Human sera samples used for the EEEV studies were collected between January 2003 to December, 2010. Twenty-two samples were pre-selected, of which 10 were negative. Due to the low quantity of clinical EEEV antibody positive serum samples, contrived samples were created by making a 1:2 dilution containing negative alphaviral antibody serum (positive flaviviral antibody serum) as the diluent. Therefore, a total of 20 presumptive positive EEEV antibodies (10 original presumptive positive and 10 contrived samples) and 10 negative (flaviviral antibody positive) sera samples were used for this segment of the validation.

The human serum samples for WNV antibodies were obtained from January, 2015 to December, 2016. Thirty-eight human samples were pre-selected. Out of these 38 human serum samples, 20 positive samples met the P/N criterion and a total of 10 negative samples met the volume requirement. All human samples were initially stored in -80°C freezers, however, upon use, samples were maintained at 2-8°C. All avian samples were stored at 2-8°C.

Plaque Reduction Neutralization Test. Plaque reduction neutralization test was used to quantify the titer of neutralizing antibodies for a virus with the purpose of diagnosing infections. All PRNT assays were performed at BPHL-Tampa. Neutralization assays for human sera samples were performed in a 12-well plate according to CDC/DVBD standard protocol. Neutralization assays for avian samples were performed in a 6-well plate according to standard BPHL protocol.

Each sample was simultaneously tested with the wild type virus and the corresponding chimera virus, for EEEV and WNV. Samples were diluted 1:5, with the diluent being SVD supplemented with 8% FCS and then heat inactivated at 56°C for 30 minutes to destroy any complement and to inactivate viruses. Samples then were serially diluted (i.e. two-fold) twelve times for human sera and three times for avian sera in a 96-well Tissue Culture Falcon plate to achieve endpoint 90% neutralization titers (CDC/DVBD, 2017; Rabe et al., 2016). The range in titers for human sera using EEEV and WNV was 1:10 to 1:20480, with the lowest titer being 1:10. The titers for avian sera were 1:10 to 1:40. The viral challenge dilution was placed onto the serially diluted sera samples, creating a final dilution of 1:2. The viral-serum mixtures were incubated overnight at 4°C or for 1 hour at 37°C in a 5% CO_2 incubator. Following incubation, media was aspirated from the culture plates and 100 µl of the viral-serum mixture was inoculated onto the monolayer of Vero cells. The plates then were incubated at 37°C in a 5% CO_2 incubator

for one hour and rocked every 15 minutes. An overlay of media containing supplemented EMEM (as described above) was placed onto the Vero cell, allowed to harden, and placed into the incubator. A second overlay media containing 2% of neutral red was placed onto the plates two to four days post the first overlay for EEEV and WNV, respectively. The plaques then were counted by hand and recorded for two days after the second overlay. Titer wells that contained too many plaques were considered too numerous to count (TNTC). For each PRNT, a back titer was run to ensure that the viral concentrations for both the wild type and chimera viruses were appropriate and met the 200 plaque challenge, which is a control that was established to measure the parameters of the assay (CDC/DVBD, 2017). Neutralizing antibody titers were calculated as the reciprocal of the highest serum dilution that reduced the virus plaque count by 90% for human sera samples (Johnson et al., 2011; Rabe et al., 2016). Avian sera samples used a 95% reduction in order to compensate for the low volume of sera sample given. A $PRNT_{90}$ titer of 10 or greater was considered positive. Specimens were tested once, unless noted otherwise.

Statistical analyses. A nonparametric test of Cohen's kappa was used to assess the level of agreement between positive and negative results of the wild type and the chimera virus (Johnson et al., 2011). A contingency table of the binary serostatus results was used to determine the sensitivity and specificity of each chimera virus using the wild type virus-based PRNT as the gold standard (Komar, Langevin, & Monath, 2009). This answers the research question of whether the chimera viruses are an efficient alternative to the wild type viruses (Johnson et al., 2011; Komar, Langevin, & Monath, 2009). Wilcoxon Signed Rank Test and kappa statistics was used to assess the level of agreement between the positive titer results obtained from the wild type virus and the chimera virus (Gennaro, Lorusso, Casaccia, Conte, Monaco, & Savini, 2014). This answers the research question of whether the chimera virus yields similar titers to the

corresponding wild type virus (Gennaro, Lorusso, Casaccia, Conte, Monaco, & Savini, 2014; Johnson et al., 2011). The kappa statistical interpretations are as follows: a value of less than 0.20 is a poor agreement, 0.21 to 0.40 is a fair agreement, 0.41 to 0.60 is a moderate agreement, 0.61 to 0.80 is a good agreement, and 0.81 to 1.00 is a very good or excellent agreement (McHugh, 2012). A ratio containing the obtained kappa values for avian to human serum samples were calculated to illustrate the variations of titer agreements between the two specimen sources. Additionally, a Pearson's r correlation statistics was performed to demonstrate the overall degree of association among titers. The level of significance was set to alpha (α) at 0.05. All of the statistical analyses were performed using SPSS version 23.

Ethical Considerations. For this study, no patient contact was needed and samples were de-identified and no personal identifiers were recorded. The Department of Health protects the confidentiality of all persons who may have had arboviral diseases (Ch.381.0055, F.S.; BPHL, 2014). This study was approved by both the USF IRB (Pro00031338) and the DOH IRB (0004682).

Chapter Four: Results

Eastern Equine Encephalitis PRNT

Serostatus of Avian Samples. Analyses of the avian sera test for EEEV antibodies demonstrated that both the wild type and chimera challenge virus were neutralized by antibodies present in the sera two to three days post inoculation. A high concordance of the binary results (positives versus negatives) for the serostatus of the avian sera samples was observed between wild type and chimera virus-based PRNT assay. There was a very good agreement ($\kappa = 0.92$, $p < 0.0005$), between the wild type and the chimera regarding the serostatus of the avian sera. The results furthered illustrated that the SINV/EEEV had 100% sensitivity and 90% specificity when confirming avian sera samples. Both the wild type and the chimera virus were able to confirm all 20 presumptive positive samples, however, the chimera virus obtained an additional positive result when the wild type virus confirmed it as negative (**Table 1**).

Table 1. **Contingency table for the EEEV Avian sera samples.** This table illustrates that both the wild type and the chimeric virus confirmed all 20 presumptive positive samples, however, the chimera virus obtained an additional positive result when the wild type virus confirmed it as negative. Therefore, chimera virus has 90% sensitivity and 100% specificity when confirming avian sera samples.

	EEE-WT Positive	EEE-WT Negative	Total
EEE-Chi (Positive)	20	1	21
EEE-Chi (Negative)	0	9	9
Total	20	10	30

Titer agreement of Avian Samples. The level of agreement between endpoint titers was 0.77 ($p < 0.0005$) with a positive correlation of 0.87 (Pearson's correlation, $p < 0.0005$). The overall endpoint titers for the chimera virus were comparable by 86% (26/30) to the wild type titers. Out of the 20 avian sera samples that were confirmed positive with the wild type virus, 80% (16/20) were confirmed with an equivalent titer using the chimera virus. Two of the four samples that differed had a 4-fold difference in titer, while the remainder had a 2-fold difference (**Table 2**). A Wilcoxon signed rank test revealed that there was no statistical significance between the titers of the wild type and the chimera virus and had a very small effect size ($z = -0.184$, $r = 0.024$, $p = 0.854$). For the negative avian sera samples, both the wild type and chimera virus reported equivalent endpoint titers.

Serostatus of Clinical Samples. There was a 100% serostatus agreement ($\kappa = 1$) between the wild type and chimera virus-based PRNT assays for the clinical human sera samples. One of the positive samples yielded negative PRNT results for both the wild type and chimera viruses, therefore there were a total of 19 confirmed positive samples and 11 negative samples (**Table 3**). Sensitivity and specificity for confirming human sera was both 100%.

Titer agreement of Clinical Samples. An overall lower titer concordance for human sera was observed between wild type and chimera virus-based PRNT assays. The chimera titers were in fair agreement to the wild type titers ($\kappa = 0.36$; $p < 0.0005$), even though there was an overall positive correlation of $r = 0.96$ (Pearson's correlation, $p < 0.0005$). There were eight samples (42%) where there was a 2 fold difference in titers, eight samples (42%) where there was a 4-fold or more titer difference and three samples (16%) where the titers were the same (**Table 4**). The Wilcoxon signed ranked test indicated that there was a statistical significance

between the wild type and chimera titers and a medium effect size ($z = -3.574$, $r = 0.46$, $p < 0.0005$).

Table 2. Comparison of neutralizing antibody titers in sera from infected sentinel chickens using wt and chimera challenge EEEVs in $PRNT_{90}$

Sample type	wt-EEEV	SINV/EEEV
Avian		
1	>40	>40
2	>40	>40
3	>40	>40
4	<10	>40
5	>40	1:10
6	<10	<10
7	>40	>40
8	>40	>40
9	1:20	1:20
10	1:20	1:10
11	<10	<10
12	<10	<10
13	<10	<10
14	<10	<10
15	<10	<10
16	>40	>40
17	1:20	>40
18	>40	>40
19	>40	>40
20	>40	>40
21	>40	>40
22	1:10	1:10
23	>40	>40
24	>40	>40
25	>40	>40
26	>40	>40
27	>40	>40
28	<10	<10
29	<10	<10
30	<10	<10

Table 3. **Contingency table for the EEEV Clinical sera samples.** This table shows that both the wild type and chimera virus confirmed all twenty presumptive positive samples. One sample confirmed as negative for both the wild type and chimera virus, therefore there was a total of 19 confirmed positive samples and 11 negatives. The chimera virus has 100% sensitivity and 100% specificity when confirming avian sera samples.

	EEE-WT Positive	EEE-WT Negative	Total
EEE-Chi (positive)	19	0	19
EEE- Chi (negative)	0	11	11
Total	19	11	30

West Nile Virus PRNT

Serostatus of Clinical Samples. Clinical samples used in the WNV assay were in excellent agreement ($\kappa = 1.00$) between the chimera virus and the wild type virus regarding the serostatus. The YF/WN chimera virus had a 100% sensitivity and specificity. Similar to the EEEV PRNT, one of the 20 presumptive positive samples confirmed as negative for both the wild type and chimera virus, therefore there were a total of 19 confirmed positive samples and 11 negatives (**Table 5**).

Titer agreement of Clinical Samples. The titers of clinical samples between the chimera and the wild type obtained a positive correlation ($r = 0.98$, $p < 0.0005$, Pearson's correlation), yet, reached a fair measurement of agreement ($k = 0.31$; $p < 0.0005$). The chimera virus had 58% (11/19) of the confirmed positive samples that were in 2-fold titer difference and 32% (6/19) that were a 4-fold or more titer difference (**Table 6**). The Wilcoxon signed ranked test demonstrated that there was a statistical significance between the wild type and chimera titers with a medium effect size ($z = -3.745$, $r = 0.48$, $p < 0.0005$).

Table 4. Comparison of neutralizing antibody titers in sera from diagnostic infected humans using wt and chimera challenge EEEVs in $PRNT_{90}$

Sample type	wt-EEEV	SINV/EEEV
Human		
1	1:20480	1:2560
2	1:1280	1:1280
3	1:160	1:80
4	1:80	1:40
5	1:20480	1:5120
6	1:320	1:20
7	<10	<10
8	1:160	1:40
9	1:2560	1:320
10	1:10	1:10
11	<10	<10
12	<10	<10
13	<10	<10
14	1:20480	1:10240
15	1:10240	1:5120
16	1:20	1:10
17	1:320	1:320
18	1:10240	1:1280
19	1:160	1:10
20	1:640	1:80
21	1:80	1:40
22	1:40	1:20
23	1:20	1:10
24	<10	<10
25	<10	<10
26	<10	<10
27	<10	<10
28	<10	<10
29	<10	<10
30	<10	<10

Table 5. **Contingency table for the WNV Clinical samples.** This table demonstrates that both the wild type and chimera virus confirmed all twenty presumptive positive samples. One sample confirmed as negative for both the wild type and chimera virus, therefore there was a total of 19 confirmed positive samples and 11 negatives. The chimera virus has 100% sensitivity and 100% specificity when confirming avian sera samples.

	WNV-WT Positive	WNV-WT Negative	Total
WNV-Chi (positive)	19	0	19
WNV-Chi (negative)	0	11	11
Total	19	11	30

Table 6. Comparison of neutralizing antibody titers in sera from diagnostic infected humans using wt and chimera challenge WNVs in $PRNT_{90}$

Sample type	wt-WNV	YFV/WNV
Human		
1	1:640	1:320
2	1:640	1:320
3	1:1280	1:320
4	1:1280	1:640
5	1:1280	1:320
6	1:2560	1:1280
7	<10	<10
8	1:320	1:160
9	<10	<10
10	1:640	1:320
11	<10	<10
12	1:2560	1:1280
13	1:1280	1:1280
14	1:2560	1:1280
15	1:5120	1:640
16	1:2560	1:1280
17	1:2560	1:1280
18	1:80	1:80
19	1:80	1:20
20	1:40	1:20
21	1:2560	1:320

22	1:40	1:10
23	<10	<10
24	<10	<10
25	<10	<10
26	<10	<10
27	<10	<10
28	<10	<10
29	<10	<10
30	<10	<10

Analyses of Prior Studies

Additional statistical analyses were performed on data from two studies that were previously performed at the FDOH, BPHL-Tampa. One study standardized and validated the West Nile Chimera in PRNT for the purpose of diagnosis avian serum samples. The sample criteria and the number of positive and negative samples are the same as in this study. The second study standardized and validated St. Louis encephalitis chimera in PRNT for diagnosis of avian and clinical human serum samples. The sample criteria for both the avian and human serum samples were the same as in this study.

West Nile Virus

Serostatus of Avian Samples. The YF/WNV chimera was able to neutralize in a like manner to the wild type virus in the avian l sera samples Avian samples tested for WNV were in excellent agreement ($\kappa = 1.00$) between the chimera virus and the wild type virus regarding the serostatus. The YF/WN chimera virus had a 100% sensitivity and specificity.

Titer agreement of Avian Samples. The titer agreement between the chimera and the wild type virus was 0.77 ($p < 0.0005$) with a positive correlation of 0.94 ($p < 0.0005$, Pearson's correlation). The overall endpoint titers for the chimera virus were comparable by 86% (26/30) to the wild type titers. Out of the 20 avian sera samples that were confirmed positive with the

wild type virus, 80% (16/20) were confirmed with an equivalent titer using the chimera virus. All four samples had a 2-fold difference in titer. The Wilcoxon signed ranked test illustrated that there was no statistical difference between the titers of the wild type and the chimera virus and had a very small effect size ($z = -0.368$, $r = 0.067$, $p = 0.713$). For the negative avian sera samples, both the wild type and chimera virus reported equivalent endpoint titers.

St. Louis Encephalitis Virus

Serostatus of Avian Samples. The chimera YF/SLE was able to neutralize in a like manner to the wild type virus in the avian and clinical sera samples. The avian samples obtained a good serostatus agreement between chimera and wild type virus ($\kappa = 0.67$). The YF/SLE chimera had a specificity of 100%; however the sensitivity was 75%. In the wild type virus-based PRNT, five out of the 30 avian samples were detected as negative whereas, the samples that were detective as positive in the chimera-based PRNT. As before, one of the 20 presumptive positive samples confirmed as negative for both the wild type and chimera virus, therefore there were a total of 19 confirmed positive samples and 11 negatives.

Titer agreement of Avian Samples. The avian titer agreement between the chimera and the wild type virus was 0.67 ($p < 0.0005$) with a positive correlation of 0.69 ($p < 0.0005$, Pearson's correlation). The overall endpoint titers for the chimera virus were comparable by 83% (25/30) to the wild type titers. Out of the 20 avian sera samples that were confirmed positive with the wild type virus, 26% (5/19) were confirmed with an equivalent titer using the chimera virus. A total of 32% (6/19) of the avian sera samples had a 4-fold titer difference and 16% (3/19) obtained a 2-fold titer difference. The Wilcoxon signed ranked test illustrated that there was no

statistical significant difference between the titers of the wild type and the chimera virus and had a very small effect size ($z = -0.64$, $r = 0.012$, $p = 0.949$).

Serostatus of Clinical Samples. The human clinical samples tested for SLEV were in excellent agreement ($\kappa = 1.00$) between the chimera virus and the wild type virus regarding the serostatus. The YF/SLE chimera virus had a 100% sensitivity and specificity with respect to human sera samples. There were a total of nine clinical samples that were tested which consisted of four positive samples and five negative samples.

Titer agreement of Clinical Samples. The titer agreement for SLEV clinical sera samples were fair between the chimera and the wild type virus ($\kappa = 0.38$; $p < 0.0005$) with a positive correlation of 0.97 ($p < 0.0005$). The overall endpoint titers for the chimera virus were 77% (7/9) comparable to the wild type titers. Out of the four clinical sera samples that were confirmed positive with the wild type virus, 50% (2/4) were confirmed with an equivalent titer using the chimera virus. A total of 25% (1/4) of the clinical sera samples had a 4-fold titer difference and 25% (1/4) obtained a 2-fold titer difference. The Wilcoxon signed ranked test illustrated that there was no statistical significant difference between the titers of the wild type and the chimera virus and had a moderate effect size ($z = -1.342$, $r = 0.24$, $p = 0.183$).

Chapter Five: Discussion

Assessment of the Serostatus Outcomes

The purpose of this study was to assess and validate the use of chimera viruses in PRNT for diagnostic purposes for the state of Florida. The PRNT results for EEEV, WNV, and SLEV chimeras were comparable to their respective counterpart wild type viruses for the avian and clinical serum samples. The avian sera samples that were tested for EEEV antibodies had a 92% serostatus agreement between the wild type virus and the chimera virus. The 92% serostatus agreement was due to one additional positive sample that the chimera resulted than the wild type virus. For the EEEV clinical serum samples, there was a 100% serostatus agreement between the wild type and the chimera virus. There was a 100% serostatus agreement between the wild type virus and the chimera virus for both avian and clinical samples tested for WNV antibodies. The YF/SLE chimera performed analogous to wild type virus. The serostatus agreement for the avian samples was 67%. The decrease in serostatus agreement may be attributed to pipetting errors that could have occurred while creating the viral-serum mixture. Nonetheless, there were no errors that caused a significant impact to induce a change in results. However, there was a 100% serostatus agreement between the wild type virus and the chimera virus for the clinical samples tested for SLEV antibodies.

The serostatus results indicate that the non-EEEV, non-WNV, and non-SLEV neutralizing antibodies in the sera reacted effectively to both the wild type virus and the chimera virus for each virus, respectively. Moreover, antibody recognition and neutralization are specific to the EEEV, WNV, and SLEV structural proteins of the chimera virus, respectively (Johnson et al., 2011). The observed outcome confirms that the avian and clinical sera did not contain

antibodies against alphavirus (i.e. SINV) nonstructural proteins and flavivirus (i.e. YFV) nonstructural proteins. Therefore, there was no evidence that SINV or YFV nonstructural protein genes present in each chimera influenced serological results (Johnson et al., 2011).

Assessment of the Titer Agreements

A notable difference in PRNT endpoint titers were detected between the wild type and chimera viruses for EEEV, WNV, and SLEV antibodies among avian and clinical samples. In all of the cases, the titer differences ranged between two-fold to four-fold or greater. However, the difference in titers is not uncommon.

Comparison of Studies. Several studies have also illustrated differences in titers between the chimera and the wild type virus. Johnson et al. (2011) reported that the log titers of antibodies to the EEEV wild type were on average 1.3 times greater than the log titers to the antibodies of the chimera. Haolin et al. (2007) also displayed that there was a one to two-fold difference in titers between the parental and the chimera virus. Komar, Langevin, & Monath (2009) documented that the titers of the WNV wild type was greater by four-fold or more when compared to the chimera. Pugachev et al. (2004) demonstrated that the titers for the SLE chimera virus were up to four times lower than the wild type virus.

Hypotheses for Difference in Titer Agreements. The reason behind the differences in titers is not well understood. One possible reason for this difference is that the construct of the chimera appears to improperly bind to all the receptors of the antibodies due to conformational changes in the chimera virus envelope, thus decreasing the sensitivity, in comparison to the wild type that may bind to several receptors creating a greater sensitivity (Johnson et al., 2011; Komar, Langevin, & Monath, 2009). Theoretically, differences in titer agreements should be

very minute since the structural protein compositions of these chimera viruses should be analogous to the wild type (Johnson et al., 2011). One probable explanation for the difference may be that a conformational change in the chimera virus envelope could have lowered the avidity or blocked the receptors that recognize certain immunoglobulins (Komar, Langevin, & Monath, 2009).

Another possible explanation for the reduction in titer sensitivity is that the RNA interactions among the proteins impact the structure of the viruses in such a way to induce a slight change in conformation in the chimera viruses that results in titer differences (Johnson et al., 2011). The conformational change may be due to abnormal interactions between the vaccine portion (or SINV in the case of EEE chimera) of the chimera RNA genome and the viral proteins (Johnson et al., 2011; Komar, Langevin, & Monath, 2009). In order to test these hypotheses, a structural study of the chimera viruses and the corresponding wild type virus would have to be conducted via cryo-electron microscopy (Johnson et al., 2011).

Furthermore, the differences within the titers could occasionally result in a sample that is confirmed as weakly positive (low titer) using the wild type virus, whereas the sample would be confirmed as negative using the chimera virus (Johnson et al., 2011). However, this seroconversion is also dependent upon the time of collection of the sera specimens. Sera samples that are collected too early or too late with regards to onset of symptoms can cause the titers to fall below the lowest titer of quantification using the chimera viruses and result in false negatives (Beaty, Calisher, & Shope, 1995; Johnson et al., 2011). Nevertheless, naturally occurring infections typically result in titers well above the lower titer of quantification and therefore should not yield false negatives. Moreover, the relatively small possibility of false negatives

occurring is override by the enhanced safety component of the chimera viruses and the reduction in regulatory requirements for using chimeras (Jonshon et al., 2011).

Ratio of Kappa Values. Interestingly, upon observing the differentiation among titer agreements between the chimera viruses and the wild type viruses, a pattern was noticed. The titers obtained from the avian sera samples had a higher concordance to the titers of the wild type virus when compared to the titers obtained from the clinical sera samples. The variation in titer agreements among the avian and clinical sera samples was illustrated amid the EEE, WN, and SLE chimera viruses.

The titers of avian sera that were tested for EEEV were typically 2.14 times more in agreement with wild type titers than the clinical sera. Again, the titer agreement for the EEEV antibody positive avian sera samples was 0.77, whereas the titer agreement for the EEEV antibody positive clinical samples was 0.36. Analogous to EEEV, the titers of the avian sera sample were, on average, 2.48 times more in agreement with the wild type virus titers than the clinical sera for WNV. To reiterate, the titer agreement for the WNV antibody positive avian sera samples was 0.77, meanwhile the clinical sera obtained a titer agreement of 0.31. The comparison of avian to clinical titer agreements for SLEV was not as drastic as EEEV and WNV. For SLEV, the avian titer agreement for positive sera samples was generally 1.76 times more in agreement with the wild type virus titers than the clinical sera. The titer agreement for the avian sera samples was 0.67; however, the titer agreement for the clinical sera was 0.38.

Comparison of Studies. This pattern has also been cited in other studies as well. Komar, Langevin, & Monath (2009) study demonstrated that the WNV titer agreement among avian samples was 0.86, whereas the titer agreement for the equine samples was only 0.45. Johnson et al. (2011) also displayed similar titers between the wild type and the EEE chimera virus among

the murine samples (mice had less than an 8-fold titer difference), but obtained varying titers among the equine (equines had a 4 to 16 fold titer difference) and the clinical samples (humans had a 4 fold or greater titer difference) within their tables. In addition, there are other studies that illustrate a variation among interspecies in humoral immune responses such as mice and humans and mice and horses (Komar, Langevin, & Monath, 2009; Oliphant et al., 2007, Sanchez et al., 2007).

Hypothesis for Difference in Kappa Values. One likely explanation for the differentiation of titer agreements between avian sera samples and clinical sera samples is that the supplement added to the diluent that is used throughout PRNT does not support clinical or equine sera samples and therefore yields a lower sensitivity than the avian or murine sera samples when performing PRNT with the chimera virus (Komar, Langevin, & Monath, 2009; Even, Sandusky, & Barnard, 2006). In this study, as well as the several studies mentioned above, the supplement added to the diluent of choice was Fetal Bovine/Calf Serum. Animal sera typically consist of prions, mycoplasmas, fungi, bacteria, hormones and endocrines, and viruses. Fetal Bovine/Calf Serum has several facets that can hinder the results of an experiment (Even, Sandusky, & Barnard, 2006; Jochems, van der Valk, Stafleu, & Baumans, 2002). Another possible explanation for the difference in titer agreements between avian and clinical sera samples has to do with how the test is performed. Although, the media is poured or vacuumed off of the monolayer of cells, the cells are never washed with a buffer (i.e. Phosphate Buffer Saline, PBS). In cell culture, it is highly important to wash with PBS so that the pH and the osmolality of the cells can be maintained. The buffer also aids in the removal of inhibiting growth factors found within FBS/FCS (Dulbecco & Vogt, 1954). Furthermore, there is an additional 50 µl of FBS/FCS supplemented media that remains on the monolayer of cells.

Commercially purchased FBS/FCS has been reported to be between 20% to 50% virus positive. Removal of these viral contaminants can be difficult and costly. Theses viral contaminants can introduces adverse factors that can potentially skew the outcome of experiments performed, such as PRNT. Another factor that can influence the results of an assay is the FBS/FCS ability to interfere with genotypic and phenotypic cell stability (Even, Sandusky, & Barnard, 2006). In addition, commercially purchased serum can vary quantitatively and qualitatively in their composition and therefore create lot-to-lot variable that may produce adverse outcomes. Moreover, serum-supplemented media may lack the ability to support the growth of specific cell types and thus hinder the performance cell-based assays (Brunner, Frank, Appl, Schoffl, Pfaller, & Gstraunthaler, 2010).

Overall, animal sera introduce a wide variety of unknown variables into tissue culture (Brunner, Frank, Appl, Schoffl, Pfaller, & Gstraunthaler, 2010; Jochems, van der Valk, Stafleu, & Baumans, 2002). The disadvantages that animal sera supplement introduces—in conjunction with the reduced sensitivity of the chimera viruses creates several possibilities that may explain the difference in titer variation between wild type virus and chimera virus in addition to avian and clinical sera samples. The animal serum supplement may be a factor in the chimera's ability to properly bind to the antibody in order to illicit a similar response to the wild type virus. Antibodies within the animal serum could bind to chimera instead of the antibodies in the specimen of interest (Brunner, Frank, Appl, Schoffl, Pfaller, & Gstraunthaler, 2010; Komar, Langevin, & Monath, 2009).

With these possibilities in mind, alternative supplements, such as serum-free media, for equine and clinical sera samples should be considered and tested to observe whether the titer agreements become more similar to the wild type virus compared to avian and murine sera

samples. The different supplements may make a difference in sensitivity for the chimeras and could enhance plaque morphology in PRNT (Appendix G). Furthermore, washing the plate of monolayer cells with PBS prior to inoculation may optimize the performance of the chimera viruses for both avian and clinical sera samples.

Limitations of the Study

There were a few limitations to performing PRNT. One of the primary limitations to performing the validation of the SINV/EEEV chimera was the limited quantity of clinical samples that were EEEV antibody positive. Since EEE is a rare arboviral infection and there were only thirteen positive samples that could be retrieved, additional samples had to be contrived in order to meet the qualifications under CLIA regulations in validating an assay, which recommends a minimum of 20 samples for performing validations. In addition, during one of the EEEV PRNT validations, phenol red was accidentally used instead neutral red as the viable dye. Though the incorrect dye was used, plaques were still visible enough to read and count the plaques and therefore did not affect the outcome of the assay.

Another limitation was that all the chimera viruses had to be titrated in order to be in the correct range of working concentrations for the challenge virus. Occasionally, the titration had to be performed several times in order to yield optimal results. Furthermore, some of the chimera viruses had to be cultured prior to performing the viral titrations. This caused a delay in validating chimera viruses, but did not hinder the results or the performance of the assay.

Another predominate limitation that manifested, occurred during the quantification of plaques. The morphology of the plaques varied for each wild type virus and their counterpart chimera virus. Some viruses did not yield clear and distinct plaques, and therefore caused

difficulty in quantitating the number of plaques. Furthermore, there were problems that occurred with certain equipment such as the incubators. One of the incubators was leaking CO_2 which caused a disruption in the amount of humidity necessary to keep the cells and plaques from drying out. This caused a few of the plates to produce a smaller size plaque. Nevertheless, the plaques were visible and clear enough to read the plates and therefore had no effect on the results. Also, two different titer calculations had to be used when assessing the titers for avian samples that were performed using and endpoint of 95% for PRNT, instead of using the endpoint titer of 90% for PRNT that is frequently used for clinical samples. Therefore, the titer calculations had to be recalculated for avian clinical samples.

Conclusion of the Study

All in all, the findings of this study will provide the opportunity for public health laboratories that do not have a high-level biosafety facility or Select Agent certification to perform confirmatory diagnostic testing and provide a safer environment for laboratory personnel to perform arboviral PRNT (Komar, Langevin, & Monath, 2009). These chimeras will allow PRNT to be performed in a BSL-2 biocontainment laboratory. The use of chimera viruses serves as a sufficient surrogate diagnostic reagent in place of the wild type virus for PRNT assays when performed with nonhuman vertebrate sera and clinical sera, with the caveat of the chimera viruses having a slight reduction in sensitivity for weakly positive sera samples with low levels of neutralizing antibodies (Komar, Langevin, & Monath, 2009).

Furthermore, these findings will provide other public health laboratories that perform PRNT with more insight and understanding about chimera viruses in serological assays. The information gathered should inspire the desire to optimize the PRNT assay for each public health

laboratory that performs this assay. Alternative laboratory techniques to be used for diagnostic purposes that would have the potential to replace the classical PRNT assay are encouraged. Overall, arboviral surveillance and prevention will be greatly enhanced with the use of these chimera viruses.

Chapter Six: Future Improvements and Research

Future Improvements

Problematic Encounters. Even though PRNT is a gold standard confirmatory test, there is a lack of standardized protocols on how to properly perform PRNT. For this reason, there exists significant variation in diagnostic results across laboratories that perform this assay. Although there are only a few states within the U.S. that perform PRNT, every laboratory has their own "in house" protocol. Each laboratory uses their own cell line, media supplements, and varying types of vital dyes. All of these differences can drastically affect the outcome of the assay. Furthermore, variability exists within the methodology of counting plaques which can lead to discrepancies in results (both within and between laboratories).

These discrepancies can result in one patient sample having a strong titer for the virus in one laboratory, whereas another laboratory may view the serum sample as negative or weakly positive. For example, all of the laboratories that participated in the CDC 2017 Arbovirus Proficiency had a vastly different starting and ending titers in their assay. This resulted in drastically different titers for the unknown samples. With the numerous possibilities for discrepancy and variability, the state and local public health laboratories need more guidance and structure for performing PRNT for diagnostic purposes.

Possible Solution. With these variances taking place, future improvements to optimize and standardize the assay should be considered. The CDC should host a training course for PRNT in order to ensure that there is a level of standardization in how this technique is to be performed. ArboNet or another arbovirus surveillance division of the CDC should consider

developing a standardize protocol in addition to hosting a PRNT training course for those who perform diagnostic and surveillance PRNT.

Future Research

Nonetheless, there are other alternatives assays to PRNT that are less stringent and tedious to perform that have a more standardize protocol. One alternative to PRNT is the Fluorescence-based plaque reduction micro neutralization assay (PRMN). This assay consists of visual and automated readouts using enhanced green fluorescent protein that is expressed within recombinant bio-engineered viruses (Fujino et al., 2007; Duprex, McQuaid, Hangartner, Billeter, & Rima, 1999). The assay is requires less time to complete and is less labor-intensive and is also less expensive than the classic PRNT. The assay takes only two days to complete unlike seven days and uses a micro format. The PRMN has been tested and reported to yield similar values as the gold standard PRNT, and therefore should be considered as an alternative to PRNT (Haralambieva, Ovsyannikova, Vierkant, & Poland, 2008).

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Appendix A

Plaque Morphology

Eastern Equine Encephalitis Plaque formation. Plaques were visible upon two days post inoculation. The morphology of the plaques for both the wild type and the chimera were typically considerably large in size, very clear to see, and were evenly distributed among the tissue culture plate. However, due to the considerably large plaque size, the border of the plaques were not as defined which made it slightly difficult to determine where a plaque started and where it ended. Nonetheless, this was consistent among the plates for both the wild type and chimera and therefore did not affect the plaque count. Both the wild type and the chimera seem to grow fast which indicates how virulent the virus can be.

West Nile Virus Plaque Formation. Plaques were made visible for the wild type approximately two to three days post inoculation, whereas the plaques for the chimera virus typically were seen by day two post inoculation of the monolayer of cells. The morphology of the plaques for both the wild type and chimera virus were moderate in size, clear to see, however the border of the plaques were not as defined. The WNV chimera and the WNV wild type virus are not as virulent as the EEE chimera and the EEE wild type virus. There was an even distribution of plaques placed about the plate.

Appendix B

USF
UNIVERSITY OF
SOUTH FLORIDA

RESEARCH INTEGRITY AND COMPLIANCE
Institutional Review Boards, FWA No. 00001669
12901 Bruce B. Downs Blvd., MDC035 • Tampa, FL 33612-4799
(813) 974-5638 • FAX (813) 974-7091

7/14/2017

Alexis LaCrue, Ph.D.
USF Global Health
3720 Spectrum Blvd., Suite 304
Tampa, FL 33612

RE: **Not Human Subjects Research Determination**

IRB#: Pro00031338

Title: Validation of Chimeric Viruses in Plaque Reduction Neutralization Tests for Arboviral Disease Diagnostics

Dear Dr. LaCrue:

The Institutional Review Board (IRB) has reviewed your application. The activities presented in the application involve methods of program evaluation, quality improvement, and/or needs analysis. While potentially informative to others outside of the university community, study results would not appear to contribute to generalizable knowledge. As such, the activities do not meet the definition of research under USF IRB policy, and USF IRB approval and oversight are therefore not required.

While not requiring USF IRB approval and oversight, your study activities should be conducted in a manner that is consistent with the ethical principles of your profession. If the scope of your project changes in the future, please contact the IRB for further guidance.

If you will be obtaining consent to conduct your study activities, please remove any references to "research" and do not include the assigned Protocol Number or USF IRB contact information.

If your study activities involve collection or use of health information, please note that there may be requirements under the HIPAA Privacy Rule that apply. For further information, please contact a HIPAA Program administrator at (813) 974-5638.

Sincerely,

V Jorgensen MD

E. Verena Jorgensen, M.D., Chairperson
USF Institutional Review Board

Appendix C

Mission:
To protect, promote & improve the health of all people in Florida through integrated state, county & community efforts.

Florida HEALTH

Vision: To be the **Healthiest State** in the Nation

Rick Scott
Governor

Celeste Philip, MD, MPH
Surgeon General & Secretary

INSTITUTIONAL REVIEW BOARD NON-RESEARCH DETERMINATION

July 25, 2017

To: Alexis LaCrue, Ph.D.

Protocol Title: Usage of Chimeric Viruses in Plaque Reduction Neutralization Tests for Flaviviral and Alphaviral Disease Diagnostic

Review Type: Staff

Based on the information provided, the Ethics and Human Research Protection Program determined your activity is not research as defined in DOH policy and federal regulation, to mean "systematic investigation…designed to develop or contribute to generalizable knowledge" (§ 45 CFR 46.102(d)).

If the design of the project changes, so that it might become systematic, or generalizable, then it is the responsibility of the researcher to submit the project for review by the DOH IRB. If you have questions about whether your activity may require IRB approval, please contact the human research protection program office so we may determine whether the additional activities come under the category of research.

If you have questions, want to offer suggestions, or talk with someone about this or other projects, please contact Rotanya Bryan or Bonnie Gaughan-Bailey at the Department of Health IRB at (850) 245-4585 or toll-free in Florida (866)-433-2775.

Thank you for your cooperation with the IRB.

Sincerely,

Bonnie Gaughan-Bailey, MPA, ASQ-CQIA
Administrator
Biomedical Research Section
Public Health Research

Federal Wide Assurance#: 00004682

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Division of Community Health Promotion
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