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Biology And Epidemiology Of Dengue Viruses In The El Paso And The Surrounding Usa And Mexico Border Communities

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BIOLOGY AND EPIDEMIOLOGY OF DENGUE VIRUSES IN THE EL PASO AND THE SURROUNDING USA AND MEXICO BORDER COMMUNITIES

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Dedication

I dedicate this dissertation to my family: My wife Jeanette and my son Marcelo, and my parents, Maria and Prudencio. To them for their unconditional support and love and encouraged me to continue this work.

BIOLOGY AND EPIDEMIOLOGY OF DENGUE VIRUSES IN THE EL PASO AND THE SURROUNDING USA AND MEXICO BORDER COMMUNITIES

by

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DISSERTATION

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Abstract

Dengue is considered one of the most important human arboviral diseases worldwide. Dengue cases have increased in the last three decades in the Americas, especially along the United States (US)- Mexico (MX) border region, where the main vector of dengue, *Aedes aegypti* mosquito, is widely distributed. Explosive outbreaks of dengue in northern Mexico have been associated with imported and local transmission of dengue in the US border communities, especially in the Southeast Texas area, also known as Rio Grande Valley. Due to the unknown if dengue virus (DENV) is present in other areas in the US border located west of the Rio Grande Valley, the main aim of this dissertation is to understand the biology and epidemiology of dengue virus in El Paso and surrounding US and MX border communities. The first aim was to determine if dengue is endemic in El Paso, Texas and Ciudad Juarez, Mexico border communities based on the serological evidence of DENV. A serosurvey for DENV antibodies among a cohort of humans in Anapra, Ciudad Juarez and pregnant women in El Paso, Texas was conducted between 2015 and 2016. Overall, 14.1% (11/78) and 8.3% (4/48) of past and recent DENV infection, respectively were detected in Ciudad Juarez. DENV serotype 1 neutralizing antibody was mainly detected in the seroconverted individuals which also did not have a travel history outside Ciudad Juarez, suggesting locally acquired dengue infection. By contrast, the past evidence of DENV in El Paso, Texas was 0.74% (11/1472), suggesting no autochthonous dengue transmission occurred in El Paso, Texas unlike other seroprevalence rates reported in dengue-endemic areas. The second aim was to evaluate the vector competence of *Ae. aegypti* mosquitoes from the US border region (El Paso and Rio Grande) to dengue and the effect of viral dose and temperature. El Paso and Rio Grande *Ae. aegypti* mosquitoes displayed a varied infectivity rate according to the dengue viral strain, ranging from 60% to 100% for DENV-2 (FSB 3381) and

DENV-2 (328298) strains, while the infectivity rate was significantly low (4% to 13%) for DENV-2 (16681) strain. Disseminated infection rates of DENV-2 (FSB 3381) and DENV-2 (328298) in El Paso and Rio Grande *Ae. aegypti* mosquitoes significantly increased through the incubation period and reached the highest rate (80% to 100%) at 14 days of incubation. A low transmission rate of DENV-2 (FSB 3381) and DENV-2 (328298) ranging from 2% to 3% was observed in El Paso and Rio Grande *Ae. aegypti* mosquitos' saliva. Furthermore, a lower dengue viral exposure dose (FSB 3381 and 328298) significantly decreased the infectivity rates in El Paso and Rio Grande *Ae. aegypti* mosquitoes and delayed the viral dissemination. Elevated temperatures (28°C and 32°C) increased the infectivity and viral replication of DENV-2 (FSB 3381) in El Paso and Rio Grande *Ae. aegypti* mosquito body, while disseminated infection rates differed between Rio Grande (16.6%) and El Paso (64%) mosquitoes at low temperatures (24°C). Finally, the third aim was to isolate and characterize a dengue virus from the Mexican/US border and evaluate its effects on the vector competence on the US border *Ae. aegypti* mosquitoes. A DENV-1 genotype V was isolated from a febrile patient displaying a viremia of 2.69 $\log_{10}$ PFU/mL in Ciudad Juarez in 2015. Five amino acid changes in the DENV-1 isolate were unique in comparison to other Mexican DENV isolates. No travel history was reported in the patient before the onset of illness, suggesting locally acquired dengue infection in Ciudad Juarez. Infectivity rates in El Paso and Rio Grande *Ae. aegypti* mosquitoes to DENV-1 isolate were significantly associated with the viral ingested dose. Furthermore, El Paso mosquitoes were more susceptible (dose to infect 50% mosquitoes [MID_{50}] < 4 $\log_{10}$ PFU/mL) to DENV-1 isolate than Rio Grande mosquitoes (MID_{50}= 5.23 $\log_{10}$ PFU/mL). A 4 $\log_{10}$ PFU/ml exposure dose of DENV-1 isolate was able to completely disseminate in the infected bodies of El Paso and Rio Grande mosquitoes at 14 days of incubation. The transmission rate of

DENV-1 isolate in El Paso and Rio Grande *Ae. aegypti* mosquitoes ranged from 3.23% to 5.71%. The variation in the vector competence observed in this study can be linked to genetic interactions between the viral strain and the *Ae. aegypti* strain (gene by gene interactions) and potential salivary gland barriers in *Ae. aegypti* from the US border region. Overall, this is the first study that described the epidemiology of dengue in El Paso and Ciudad Juarez border communities and the interaction between *Ae. aegypti* from the US border and DENV and contributed to its understanding in the US border region.

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Chapter 1: Background

1.1. Arbovirus

Arthropod-borne viruses (Arboviruses) refer to viruses biologically transmitted to a susceptible vertebrate host by arthropod vectors, such as mosquitoes, ticks, sand flies, and biting midges in which the viruses replicate and are then transmitted by bite to a susceptible vertebrate. Currently, there are more than 500 arboviruses worldwide registered in the International Catalog of Arthropod-Borne Viruses (Karabatsos, 1985) and about 100 are known to infect animals and humans, respectively (Hubálek et al., 2014). Human and animal diseases caused by arboviruses range from subclinical or mild to moderate febrile illness to more severe and fatal disease such as encephalitis and/or hemorrhagic manifestations.

Arboviruses are classified into five major families based on antigenic relationships, morphology, genetics, and replication mechanisms including the families *Togaviridae*, *Flaviviridae*, *Bunyaviridae*, *Reoviridae*, and *Rhabdoviridae*. As an exception, a group of arboviruses is classified in other taxa in the International Catalog of Arthropod-Borne Viruses (Karabatsos, 1985).

Arboviruses were initially named by the illness they cause, e.g., bluetongue (sheep disease), dengue, or chikungunya (break bone disease). Subsequently, the geographical location of the original isolation of the virus was adopted for use to name new arboviruses e.g., Eastern or Western equine encephalitis virus and Iquitos and Mayaro viruses (Arbovirus Names 1969). Arboviruses have circulated in vertebrate hosts for hundreds to thousands of years. Many of them are considered to be re-emerging arboviruses, such as dengue virus (DENV) and chikungunya virus (CHIKV), based on the increasing incidence and geographical spread of these viruses.

1.2. Arbovirus Transmission

The transmission of arboviruses involves hematophagous arthropod vectors that are susceptible to infection, and that serve as a host for the replication, dissemination, and transmission of the virus to a susceptible vertebrate host. There are two main transmission cycles of the arboviruses: the enzootic or sylvatic cycle involving vertebrate hosts and a rural or semi-urban arthropod vector e.g., Venezuelan equine encephalitis (VEEV), West Nile (WNV); and the urban and/or rural epizootic/epidemic cycle which has an urban/domestic vector, such as *Aedes aegypti*, and a vertebrate host, either animals and/or humans (e.g., dengue, Zika, Chikungunya). However, human infections or epidemics can occur because of spillover of viruses from the enzootic cycle e.g., yellow Fever and sylvatic dengue. In addition, some arboviruses, such as Japanese encephalitis virus (JEV) and VEEV utilize domestic animals as amplifying hosts that once infected develop very high viremia and then are available to infect mosquito vectors, thus resulting in an increase in the risk of human infection. Other variations of arbovirus maintenance and transmission cycles can occur in nature, such as vertical transmission, where the virus is directly transmitted from an infected female arthropod to its progeny e.g., LaCrosse virus, Japanese encephalitis virus, yellow fever virus (Aitken et al., 1979; Rosen et al., 1978; Watts et al., 1973).

1.3.Dengue

Dengue is caused by the mosquito-borne dengue virus's serotypes 1, 2, 3, and 4 (DENV-1 to DENV-4) of the *Flaviviridae* family, genus F*lavivirus* (Westaway et al., 1985). Dengue is the most important viral disease of humans transmitted by mosquitoes (Bhatt et al., 2013; Murray et al., 2013)and is endemic in tropical and subtropical regions including more than 100 countries in southeast Asia, America, Western Pacific islands, Africa and the Mediterranean areas.

Annually, more than 390 million of human infections occur worldwide, of which 96 million are symptomatic infections ranging from mild to severe disease. The mild form or classical dengue fever disease affects 100 million people, while the more severe forms of disease including dengue hemorrhagic fever (DHF) and shock syndrome (DSS) cause 200 to 500 thousand human cases, with a fatality rate for DHF of 5 to 50% among children under the age of 15.

According to the World Health Organization (WHO), the incidence of dengue increased by 30-fold during the past 50 years (WHO, 2012) and is considered to be an under-estimate of the true dengue disease burden in some countries, such as India, Indonesia, China, Brazil and the Africa continent due to the lack of clinical and laboratory diagnosis. Indeed, dengue outbreaks and severe cases of dengue disease in the Middle East and North Africa have recently increased as occurred in Pakistan, Saudi Arabia, Yemen, and Sudan (Humphrey et al., 2016). Furthermore, the geographical distribution of dengue is likely to increase in case the virus is introduced into new temperate areas, as occurred in Florida and France, or introduced by travelers when they import the virus from an endemic to a non-endemic dengue area.

The primary vector of dengue transmission to humans in urban settings is *Ae. aegypti* while *Ae. albopictus* is the most important secondary vector; with both species being common throughout the tropics and subtropics regions (Gratz, 2004; Gubler, 1998b, 1998a; Hotta, 1998). Also, ecological studies in West Africa and Malaysia identified a sylvatic cycle of dengue involving non-human primates as the vertebrate hosts, and arboreal tree-hole dwelling *Aedes* (*Stegomyia*) spp. mosquitoes as vectors (Diallo et al., 2003; Rudnick, 1965).

1.4. Dengue virus

Dengue virus is a positive single-strand RNA virus 10.8Kb in length, with an open reading frame (ORF) that encodes a single polyprotein cleaved into structural (envelope [E],

Membrane [M], and Capside[C]) and non-structural (NS1-NS2a, NS2b, NS3, NS4a, NS4b, and NS5) proteins. The virus contains an envelope and has an icosahedral symmetry of about 50 nm diameter.

The DENV serotypes (1-4) are genetically diverse due to high mutation rates of the RNA–dependent RNA polymerase, their replication rates, and their immense population sizes (Drake & Holland, 1999; Holmes & Burch, 2000), causing a limited identity at the amino acid level (60-75%). Furthermore, DENV of the same serotype have around 3% and 6% differences at the amino acid and nucleotide levels respectively and are phylogenetically classified in genotypes and clusters or clades (Rico-Hesse, 1990). Current phylogenies based on the DENV envelope gene sequences identified five genotypes for DENV-1 (Genotype I-V), six genotypes for DENV-2 (Asian I, Asian II, Cosmopolitan, American, Southeast Asian/American, and the Sylvatic), four genotypes for DENV-3 (Genotype I-IV), and four genotypes for DENV-4 (Genotype I-IV) (Chao et al., 2005; Goncalvez et al., 2002; Lanciotti et al., 1994, 1997; E. Wang et al., 2000; Weaver & Vasilakis, 2009).

Genetic variations are associated with viral fitness, virulence, and epidemic potential and also might influence the interaction with the host antiviral immune response and the pre-existing immune response (OhAinle et al., 2011) . These genetic variants have been implicated as the cause of the severe forms of dengue disease as reported for the DENV-2 Asian Genotype and the DENV-3 genotype III (Messer et al., 2003; OhAinle et al., 2011; Rico-Hesse et al., 1997).

1.5. Dengue in the Americas

The outbreak history of dengue in the Americas has been categorized into 4 phases (Dick et al., 2012). First, the introduction of DENV in the Americas (1600-1946). Second, the continental plan for the eradication of *Ae. aegypti* (1947-1970). Third, the *Ae. aegypti*

reinfestation (1971-1999) due to deterioration of vector control programs, and as the fourth phase, the increased dispersion of *Ae. aegypti*. As a consequence, the human cases of dengue (2000-2010) increased markedly in the Americas with the circulation of all four serotypes in 2013, e.g., in Mexico, Guatemala, Nicaragua, Colombia, Venezuela, French Guiana, Guadeloupe, Martinique, Brazil, Peru, and Argentina

The occurrence of dengue epidemics in Latin America has occurred every 4 to 5 years, with increasing numbers during the last years, and has been mainly associated with the rainy season. Cases of dengue occur during the first half of the year in Peru, Paraguay, and Brazil, while Central American countries, such as Mexico and Honduras have reported cases during the second half of the year (Zambrano & San Martin, 2014). In addition, other countries, including Colombia and Venezuela have reported cases throughout the year (San Martín et al., 2010).

All four DENV serotypes cause an estimated 40 million infections annually through-out most of Latin America, with about half of these in Brazil and Mexico (MX) alone (Bhatt et al., 2013; Burke et al., 1988; Endy et al., 2002; Gratz, 2004; Gubler, 1998b, 1998a; Guzman et al., 2013; Hotta, 1998; Rodriguez-Figueroa et al., 1995).

Since the recognition of the 4 serotypes in MX in 1995, the annual incidence of non-severe dengue has increased from 1.72/100,000 in 2000 to 14.12/100,000 in 2011, with a marked increase in the incidence of cases in close proximity to the United States (U.S.) border (Brunkard et al., 2007; Carrillo-Valenzo et al., 2010; CDC, 1987; Ramos et al., 2008). The reason for the increase is not fully understood but most likely reflects the circulation of multiple DENV serotypes, an increase in clinical recognition and the establishment of the national dengue surveillance program (Dantes et al., 2014).

1.6. Dengue in the United States

All four serotypes of DENV are endemic in urban communities of the Rio Grande Valley (RGV) of Texas that borders MX. One or more serotypes have circulated on the Texas side of the border periodically since 1980, but fewer cases of dengue have been reported on the U.S. side than on the MX side (CDC, 2007; Hafkin et al., 1982; Malison & Waterman, 1983). Most of the cases have been reported during sporadic outbreaks of dengue primarily in the urban communities of Matamoros, MX and in the Brownsville, Texas (Brunkard et al., 2007; CDC, 1996; Ramos et al., 2008; Thomas et al., 2016). The outbreaks on the Texas side were associated with concurrent dengue epidemics in Tamaulipas, MX and the presence of *Ae. aegypti* and *Ae. albopictus* (Champion & Vitek, 2014; Vitek et al., 2014). Outbreaks of dengue were also reported from 2007 to 2014 along the U.S – MX border in Sonora MX with 93 travel documented cases reported during 2014 in Yuma, Arizona with all infections apparently acquired in MX (Jones et al., 2016). Although *Ae. aegypti* inhabits the entire border region, with a sporadic distribution pattern for *Ae. albopictus*, DENV has not been reported from other communities along the U.S. - MX border region (Hahn et al., 2017).

1.7. *Ae. aegypti* and *Ae. albopictus* distribution

Ae. aegypti originated in Africa, where its ancestral sylvatic form, *Ae aegypti formosus* is still present. A hypothesis suggested that *Ae. aegypti* was introduced into the Americas during the slave trade era and then spread into tropical and subtropical regions worldwide (Brown et al., 2014) . *Ae. albopictus* mosquitoes originated in the Asian forests, with a zoophilic feeding behavior and spread to the islands of India and the Pacific Ocean (Delatte et al., 2009) and during the mid-1980s, expanded its range to Europe, and the Americas (Sprenger &

Wuithiranyagool, 1986). Currently, *Ae. aegypti* and *Ae. albopictus* inhabit a wide variety of urban and peri-urban settings in Asia, Africa, America, and the Pacific Ocean islands.

Ae. aegypti is established in several Southwest states in the U.S., including Texas, Arizona, New Mexico, and California (Hahn et al., 2017) **(Figure 1.1)**; in other southern states of Louisiana and Florida; and occasionally reported in the Mid-Atlantic states and Washington D.C. (Kraemer et al., 2015; Lima et al., 2016). Furthermore, *Ae aegypti* is among the most abundant mosquito species in the U.S. -MX urban border communities.

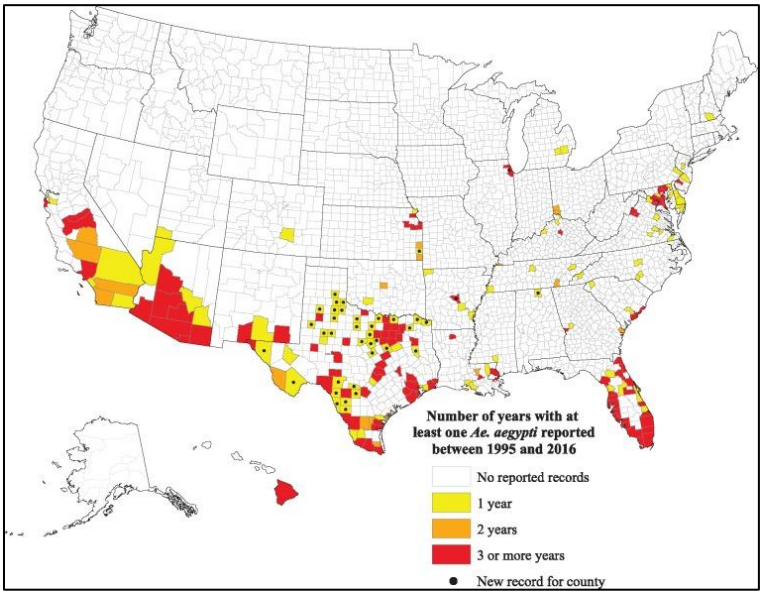


Figure 1.1. Reported occurrence of *Ae. aegypti* by county in the United States between January 1995-December 2016 (Hahn et al., 2017)

Various areas in the U.S.-MX border have experienced mean temperatures suitable for DENV transmission for more than 3 months of the year, and some other areas have temperatures favorable for year-round or seasonal transmission, e.g., southeast Texas (Mordecai et al., 2017). Therefore, environmental conditions are favorable for the emergence of DENV, and other arboviruses transmitted by *Ae. aegypti*, including Zika virus (ZIKV) and/or CHIKV in certain areas of the U.S.-MX border.

1.8. Vector competence

Ae. aegypti and Ae. albopictus mosquitoes are the main vectors of DENV because of their mosquito vector competence. Vector competence is the biological features of a mosquito to acquire a microorganism and support its transmission to a vertebrate host (Kramer & Ciota, 2015). This biological mechanism involves a very complex and poorly understood process inside the female mosquito: infection of the mosquito after taking a viremic blood meal from a vertebrate host, subsequent replication of the virus in the mosquito midgut, dissemination of the virus to the mosquito's salivary glands, and transmission to a naïve vertebrate host during feeding to acquire a second blood meal by the mosquito (**Figure 1.2**).

The interval between the mosquito infection and viral transmission by the infected mosquito is considered as the extrinsic incubation period (EIP). Furthermore, the competence of a vector is affected by intrinsic factors of the vector (Hardy et al., 1983) , such as immunity, microbiota, genetics, physiology, and the viral genotype and dose ingested by the vector; and extrinsic or environmental factors, such as temperature, humidity, and others.

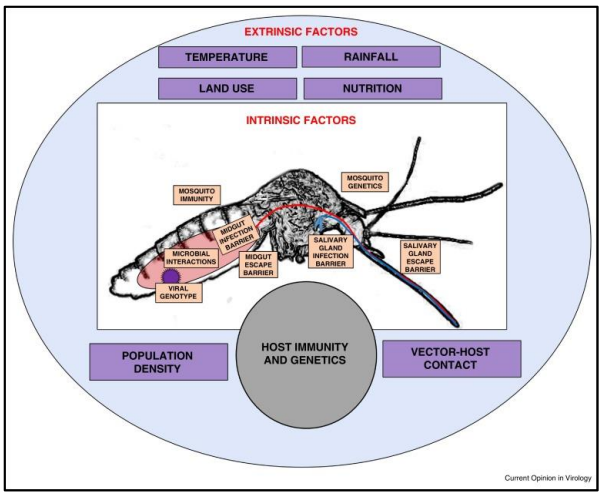


Figure 1.2. Intrinsic and extrinsic factors affecting the vectorial capacity of a mosquito vector (Kramer & Ciota, 2015)

Viral genetic changes have affected vector competence in several mosquito species e.g., WNV in *Culex spp*. (Moudy et al., 2007), and CHIKV in *Ae. albopictus* (Tsetsarkin et al., 2007) and is believed to reflect evolutionary pressures on selected mosquito populations. Also, several studies have indicated that vector competence can be affected by genotype-by-genotype interaction between the virus and the mosquito population (Fansiri et al., 2013; Pongsiri et al., 2014).

Vector competence is a component of a more complex mechanism known as vectorial capacity which incorporates other component of the vector biology and its ability to become infected and transmit the virus. These other components include vector density, probability that the vector feeds on a host per day, lifespan, and duration of the extrinsic incubation period (EIP) in the vector.

The current tendency toward global warming and the expansion of the distribution range of mosquito vectors into new temperate regions highlights the critical need to characterize mosquito vector's susceptibility to infection and their capability to transmit emerging and re-emerging arboviruses in order to improve vector control strategies and to understand the emergence of these viruses.

1.9. Main Aim, Specific Aims, and Hypothesis

Main aim

- Understand the biology and epidemiology of dengue viruses in the El Paso and the surrounding U.S. and Mexico border communities

Specific aim 1: Determine if dengue is endemic in El Paso, Texas and Ciudad Juarez, Mexico.

Hypothesis: Due to the presence of *Ae. aegypti* mosquitoes in the largest U.S border metropolis of Ciudad Juarez and El Paso and lack of passive surveillance studies for dengue infection as occurred in the Rio Grande Valley border region, a differential serological evidence of dengue infection in humans would be observed in El Paso and Ciudad Juarez border communities.

Specific aim 2: Evaluate the vector competence of *Ae. aegypti* mosquitoes from the U.S. border region for dengue virus serotype 2 and effect of the virus dose and temperature

Hypothesis: Based on the interactions between the *Ae. aegypti* strains and virus genetic characteristics (also known as genotype-by-genotype interactions), the vector competence (infection, dissemination, and transmission rates) of *Ae. aegypti* mosquitoes from the U.S border to DENV would vary with the viral strain and dose, while the temperature of incubation would affect (increase or decrease) the EIP to DENV in the U.S border *Ae. aegypti* mosquitoes.

Specific aim 3: Isolation and characterization of DENV from a patient in Ciudad Juarez and evaluate its effects on the vector competence on U.S. border *Ae. aegypti* mosquitoes

Hypothesis: Due to the increased dengue epidemics on the Mexican border and the risk of dengue transmission into the U.S border, I hypothesized that vector competence to Mexican

DENV strains can provide information if these strains can be locally transmitted by U.S border *Ae. aegypti* mosquitoes.

Chapter 2: Determine If Dengue Is Endemic In El Paso, Texas And Ciudad Juarez, Mexico

2.1. Introduction

Dengue virus (DENV) was reported to be endemic in the southeast areas of Texas that borders with the northern states of Mexico. This area also known as the Rio Grande Valley have reported occasional dengue outbreaks in the last 30 years, primarily in the border communities of Brownsville, Texas and Matamoros, Mexico (Brunkard et al., 2007; Ramos et al., 2008; Thomas et al., 2016). All the outbreaks in the Rio Grande Valley were concurrent with large dengue epidemics in Mexican border communities causing not only imported dengue cases but also locally acquired dengue cases in the Rio Grande Valley due to the presence of *Ae. aegypti* mosquitoes, which also inhabits along the U.S border region. Thus far, no other U.S border community have reported locally acquired dengue cases.

West Nile virus was first recognized as a cause of human disease during 2002 in TX and has since been the cause of annual epidemics with a total of 5,254 cases from 2002 to 2016 (ArboNET, 2016; TDSHS, 2021). The first human cases of WN were reported during 2003 in El Paso, Texas, where WNV has repeatedly been isolated from *Culex. (Cx.) quinquefasciatus* and *Culex (Cx.). tarsalis*, the primary vectors of this virus in the El Paso community (Cardenas et al., 2011; Mann et al., 2013) . Estimates of cases based on passive surveillance indicated that 271 cases of WN occurred in El Paso from 2003 through 2016, with cases occurring each year during late June to early November, and peaking in August (ArboNET, 2016; Cardenas et al., 2011).

The El Paso/Ciudad Juarez community is the largest bi-national metropolitan areas in the U.S. - MX border, where humans (12,258,192 pedestrians and 19, 982, 407 personal vehicle passengers) cross the border, annually (Annual border crossing in 2015; available from URL:https://www.bts.gov/content/border-crossingentry-data). DENV and WNV mosquito vectors, including *Aedes (Ae.) aegypti* and *Cx.* mosquitoes were first reported during 2005 to be

abundant in Ciudad Juarez (de la Mora-Covarrubias et al., 2008, 2010). Both mosquito species are also abundant in El Paso area (DM Watts Unpublished data).

Anapra is one of the most impoverished unincorporated communities in Ciudad Juarez with insufficient water supply, sanitation, and waste collection (Ruiz Hernández, 2015). Therefore, water is stored in artificial containers that provide suitable mosquito breeding habitat close to human dwellings that pose a risk for DENV infection as reported previously in other U.S/ Mexican border communities in Brownsville and Matamoros (Brunkard et al., 2007). Previous studies in Anapra reported that DEN viral ribonucleic acid (RNA) was detected in *Ae. aegypti* mosquitoes in this community (de la Mora-Covarrubias et al., 2010). Also, a high population density of *Cx. quinquefasciatus* was correlated with the low-quality of housing, income, and population density in Anapra (de la Mora Covarrubias & Olivas, 2007). In the El Paso community, there have not been any reports of DENV human infections. However, infections could not be detected due to the lack of passive surveillance or asymptomatic infections. Since there has not been any reported association of DENV infections of humans, this study was conducted to determine if DENV were causing human infection in Ciudad Juarez and El Paso border communities. Additionally, due to the previous WNV activity in El Paso, human samples from El Paso and Ciudad Juarez were also tested for WNV antibodies. The chapter was divided into 2 aims:

Aim 1: Determine if there was serological evidence of DENV infection of humans in Ciudad Juarez

Aim 2: Determine if there was serological evidence of DENV infection among a cohort of the pregnant females in El Paso, Texas

2.2. Determine if there was serological evidence of DENV infection of humans in Ciudad Juarez

2.2.1 Material and Methods

2.2.1.1. Study Site

Ciudad Juarez has an area of 188 km^2 and is located in the state of Chihuahua, MX. The estimated population is 1.51 million inhabitants (INEGI, 2020). The climate is arid with an annual mean temperature of 18.3 ºC reaching extreme temperatures (41 ºC) during the summer season. The rainy season extends from July to September, with an annual precipitation of 264.5 mm (INEGI, 2015). Anapra is a neighborhood in Ciudad Juarez with an estimated population of 16,990 inhabitants, most of whom lack sanitary services (water and sewer). Also, most residents work in manufacturing plants, the main economic activity of the community.

A serosurvey was performed for DENV and WNV antibodies among a convenience subsample of humans in Anapra (**Figure 2.1**) between May and June 2015, and between November and December 2015, or during the peak activity of *Ae. aegypti* and *Culex* mosquitoes. One member of each family among 87 households was selected to participate in the survey. The first family was chosen from the geographic center (centroid) of the Anapra neighborhood. Subsequent households of separate families located between 100 to 200 meters radius were selected according to accessibility, security, and presence of inhabitants (de la Mora-Covarrubias A & Corral-Díaz R, 2011). The survey was explained to all the household members and one member per house was invited to participate in the study. The inclusion criteria included household participants > 18 years old. One member of each of the 87 houses agreed to participate in the study. When possible, two blood samples were collected from each of the participants. The first blood sample was used as a baseline to determine the DENV and WNV

antibody prevalence rate and the second sample was used to measure the incidence of seroconversion to these viruses as evidence of infection. All the participants signed a consent form approved for this survey by the Ethics Committee at the Institute of Biomedical Sciences of the Autonomous University of Ciudad Juarez (CBE-ICB-071-10-15) and all except 9 participants completed a questionnaire to provide demographic information and any history of travel outside of Ciudad Juarez. These 9 participants were excluded, and therefore a total of 78 participants were included in the study.

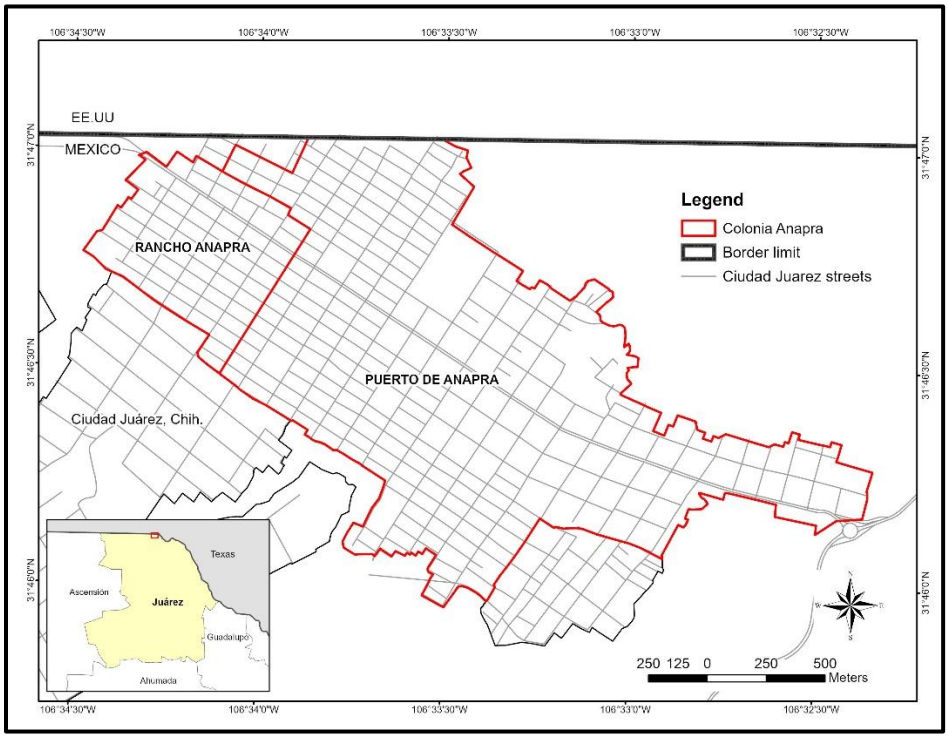


Figure 2.1. Map of Mexican border city Ciudad Juarez, Mexico highlighting the neighborhood of Anapra, Ciudad Juarez, Chihuahua -Mexico.

Blood samples were obtained by venipuncture using standard aseptic techniques with a vacutainer collection tube containing an anticoagulant. All samples were collected from each of the participants in their homes, and then placed in an Igloo container on ice packs and

transported to the laboratory. Samples were centrifuged at 1200 Gs at 4°C for 10 minutes. The plasma was removed from the packed blood cells and transferred to sterile vials and stored at -20°C until tested for IgG and neutralizing antibodies.

2.2.1.2. Indirect Immunoglobulin G (IgG) enzyme-linked immunoabsorbent assay (ELISA)

Plasma samples were tested by an indirect ELISA for IgG antibodies to DENV and WNV using lysate of infected Vero cells as an antigen coated to the bottom of the wells of 96 microtiter plates as previously described (Ansari et al., 1993). Each plasma sample was diluted 1:100 in blocking buffer (5% skim milk, 1%Tween, in Phosphate Buffered Saline [PBS] 1X pH 7.4) and tested in duplicate against a pool of DENV antigens (DENV1-DENV4) or WNV antigen and uninfected lysate cells were used as control antigen. Then, 96-well microplates coated with the cell lysates (100 µL) were incubated overnight at 4°C. The next day, microplates were washed with PBS 1X tween 0.1%, and the diluted plasma samples (100 µL/ well) were added. Then, 100 µL of a secondary antibody (Horseradish peroxidase [HRP]-conjugated mouse anti-Human IgG) was added to each well of the 96-well microplates, followed by the addition of a colorimetric substrate ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt). After incubation for 30 minutes, the optical density (OD) values at 410 nm were recorded. The cut-off OD value was calculated as the mean of six antibody-negative controls plus three times the standard deviation of the negative plasma samples (Ansari et al., 1993). Plasma samples diluted at 1:100 with an OD higher than the cut off value were considered antibody positive and then were re-tested at dilutions ranging from 1:100 to 1:6400 to determine the IgG antibody titers. Negative and positive control antibodies had a titer lesser than 1:100 and higher than 1:6400, respectively.

2.2.1.3. Immunoglobulin M (IgM)-capture ELISA to DENV

Dengue-specific IgM antibody was determined by an IgM-capture ELISA, as previously described for samples that were positive for DENV IgG antibodies (Innis et al., 1989). Briefly, 96-well microplates were coated with anti-human IgM antibody and incubated at 4°C overnight. Plasma test samples were diluted 1:100 in blocking buffer as described above for testing for IgG antibody. The next day, microplates were washed, and a volume of 100 µL of each plasma samples were added to each of the wells of the microplate. Attempts to detect IgM antibody was performed by the addition of dengue viral antigen, followed by virus-specific hyperimmune ascitic fluid and HRP-conjugated rabbit anti-mouse IgG. After adding the colorimetric substrate, OD values were read and recorded at 410 nm for each sample. The procedures for the calculation of the OD cut-off value and antibody positive samples and titers were performed as described above for WNV and DENV IgG antibodies.

2.2.1.4. Plaque reduction neutralization test (PRNT)

Plasma samples that were reactive in the indirect IgG ELISA to DENV or/and WNV were tested by PRNT to each of the DENV serotypes: DENV 1 (16007), DENV 2 (16681), DENV 3 (H87), DENV 4 (1036) and WNV (NY99) as previously described (Morens et al., 1985). Briefly, four-fold dilutions of heat-inactivated plasma samples were incubated at 4°C overnight with 30-60 plaque forming units (PFU) of DENV or WNV suspensions. The next day, mixtures of plasma/virus were inoculated on baby hamster kidney cells, clone 15 and LLCMK2 cells for DENV and WNV neutralization assays, respectively. After 3 to 7 days of incubation, cells were fixed and stained with naphthol blue-black solution. Virus dose was determined as the mean number of PFU recorded on twelve wells cells infected with 30-60 PFU based on testing of an equal volume of a dilution of the virus stock and antibody negative control human plasma.

PFUs were counted, and the dilution of plasma that reduced 80% of the virus dose was considered as the antibody titer. Samples that neutralized both DENV and WNV with titers 1:40 or higher were considered antibody positive.

Seroconversion was defined as the first plasma sample being negative for either DENV or WNV antibody and the second plasma sample from the same individual having a four-fold or higher rise in the antibody titer was considered to be confirmed as either DENV or WNV antibody.

2.2.2 Results

A total of 78 study participants provided a blood sample during the May and June 2015 survey period and 60 of the 78 participants provided samples during the November and December 2015 survey. Eighteen participants (23.1%) were not available to participate in the study after the first survey period. Most of the participants were females (66.7%), with primary education (79.5%) and 48.7% were working as housekeepers (**Table 2.1**). The results of interviewing participants during the baseline survey revealed that 30.8% (n = 24) had a history of travel outside Ciudad Juarez. All of the plasma samples positive for DENV and/or WNV IgG antibodies during the first (n = 14) and second survey (n = 17) were confirmed by PRNT. DENV IgM antibody with titers of 1:400 was detected in 2 of the 17 IgG antibody positive samples during the second survey.

The overall seroprevalence rate for DENV and WNV antibody in the first survey was 14.1% (11/78) and 5.13% (4/78) respectively; with one sample being positive for both DENV and WNV (**Table 2.2**). In the second survey, the seroprevalence of DENV and WNV antibody was 35% (21/60) and 8.33 (5/60) respectively; with one sample being positive for both DENV and WNV.

In the first survey, three of the eleven DENV antibody positives samples had neutralizing antibodies to DENV 1, 2, and 3 (**Table 2.3**). Only four participants had monotypic antibodies (one for DENV 2 and three for WNV). The remaining participants (n = 8) had polytypic responses to DENV and/or WNV.

A total of 60 participants of the 78 included in the first survey period were enrolled in the second survey period (**Table 2.3**). Among these 60 participants, 17 had neutralizing antibodies; 12 participants had the same distribution of DENV and/or WNV antibodies as during the baseline survey. Of those 12 participants, 8 had neutralizing antibody to DENV only. Among 48 participants who were negative for antibody during the first survey, 10.4% (n = 5) acquired antibody to WNV or DENV. Of these five participants, four seroconverted to DENV (one individual seroconverted to DENV 1 ($PRNT_{80}$ titer: 1:320), one seroconverted to DENV 2 ($PRNT_{80}$ titer: 1:640) and the other two seroconverted to DENV 1/ DENV 2, with DENV 1 neutralizing antibody titers 4-fold higher than the DENV 2 antibody titers), and 1 individual seroconverted to WNV ($PRNT_{80}$ titer: 1:1280) (Table 3). Also, as stated in the comparison of DENV and WNV antibody prevalence and incidence among the human cohort in Ciudad Juarez (**Table 2.4**), the 5 participants who seroconverted did not have any travel history outside Ciudad Juarez during the study. Finally, two of the four individuals who seroconverted to DENV 1 and/or DENV 2 were positive for DENV IgM antibody.

Table 2.1. Description of the 78 participants enrolled in the West Nile and dengue virus antibody 1st survey period, (May – June 2015) in Ciudad Juarez, Mexico

Sociodemographic characteristics	**N (%)**
Sex	
Male	26 (33.33%)
Female	52 (66.67%)
Occupation	
Housekeeper	38 (48.72%)
Manufacturing worker	16 (20.51%)
Merchant	12 (15.38%)
Unemployed	2 (2.56%)
Retired	2 (2.56%)
Professional	1 (1.28%)
Others	7 (8.97%)
Education	
None	4 (5.13%)
Elementary school	32 (41.03%)
Middle School	30 (38.46%)
High school	10 (12.82%)
Professional/Technician	2 (2.56%)
House construction	
Wood	9 (11.54%)
Concrete	11 (14.1%)
Mixture	58 (74.36%)
Years living in house	
0 to 5	22 (28.21%)
6 to 10	15 (19.23%)
11 to 15	11 (14.10%)
16 to 20	15 (19.23%)
>21	15 (19.23%)
Sewage	
Sewer	61 (78.21%)
Septic tank	17 (21.79%)
Travel history outside Ciudad Juarez	
Yes	24 (30.77%)
No	54 (69.23%)

Table 2.1 (Continued). Description of the 78 participants enrolled in the 1st survey period, (May – June 2015) in Ciudad Juarez, Mexico

Sociodemographic characteristics	N (%)
Age	
18-23	10 (12.82%)
24-29	6 (7.69%)
30-35	12 (15.38%)
36-41	10 (12.82%)
42-47	14 (17.95%)
48-53	8 (10.26%)
54-59	9 (11.54%)
>60	9 (11.54%)

Table 2.2. Seroprevalence rate of dengue and West Nile virus antibody among a human cohort in Ciudad Juarez, Mexico, 2015

Antibodies	**1st Period (n=78)**	**2nd Period (n=60)**
West Nile	3 (3.84%)	4 (6.66%)
Dengue	10 (12.82%)	12 (20%)
Dengue/West Nile	1 (1.28%)	1 (1.66%)
Total	14 (17.95%)	17 (28.33%)

Table 2.3. Neutralizing antibody to dengue and West Nile virus infection among a human cohort in Ciudad Juarez, Mexico, 2015

Virus	**1st period (n=78)**	**2nd period (n=60)**	**Seroconversion† ($PRNT_{80}$ titer) ***
DENV 1	0	1 (1.66%)	**1 (DENV 1, 1:320)
DENV 2	1(1.28%)	2(3.33%)	1 (DENV 2, 1:640)
DENV 1, DENV 2	2(2.57%)	4(6.66%)	**1 (DENV 1, 1:640; DENV 2, 1:80) 1 (DENV 1, 1:1280; DENV 2, 1:80)
DENV 1, DENV 3	3 (3.84%)	3 (5%)	0
DENV 1, DENV 2, DENV 3	3 (3.84%)	1(1.66%)	0
DENV 1, DENV 2, DENV 3, DENV 4	1(1.28%)	1(1.66%)	0
DENV 1, DENV 3, WNV	1(1.28%)	1(1.66%)	0
WNV	3(3.84%)	4(6.66%)	1 (WNV, 1:1280)
No antibody	64(82.05%)	43(71.66%)	-

†Seroconversion, four-fold or greater rise in antibody titer in paired sera.

*$PRNT_{80}$ titer, 80% plaque- reduction neutralization test titer.

**Number of individuals DENV IgM positive

Table 2.4. Comparison of dengue and West Nile virus antibody prevalence and incidence of seroconversion among human cohort during May to December, 2015 in Ciudad Juarez, Mexico

Variable	**% of Prevalence* (No. positive[#]/ No. tested[$])**		**% of Incidence** (No. positive[#]/ No. tested[$])**	
Sex	DENV	WNV	DENV	WNV
Male	19.23 (5/26)	3.85 (1/26)	11.11(2/18)	5.55 (1/18)
Female	11.54 (6/52)	5.76 (3/52)	6.66 (2/30)	0/30
Occupation				
Housekeeper	10.53 (4/38)	7.89 (3/38)	9.52 (2/21)	0
Manufacturing worker	12.5 (2/16)	0/16	8.33 (1/12)	8.33 (1/12)
Merchant	25 (3/12)	0/12	0/6	0/6
Unemployed	50 (1/2)	50 (1/2)	100 (1/1)	0
Professional	100 (1/1)	0/1		
Retired	0/2	0/2	0/1	0/1
Others	0/7	0/7	0/7	0/7
Education				
None	25 (1/4)	25 (1/4)	0/1	0/1
Elementary school	15.62 (5/32)	3.13 (1/32)	5.56 (1/18)	0/18
Middle School	6.66 (2/30)	6.66 (2/30)	9.09 (2/22)	4.55 (1/22)
High school	20 (2/10)	0/10	16.6 (1/6)	0/6
Professional/Technician	50 (1/2)	0/2	0/1	0/1
House construction				
Wood	22.22 (2/9)	22.22 (2/9)	20 (1/5)	0/5
Concrete	18.18 (2/11)	0/11	0/6	0/6
Mixture	12.08 (7/58)	3.45 (2/58)	8.11 (3/37)	2.7 (1/37)
Years living in house				
0 to 5	18.18 (4/22)	9.09 (2/22)	16.67 (2/12)	0
6 to 10	6.67 (1/15)	0/15	8.33 (1/12)	8.33 (1/12)
11 to 15	18.18 (2/11)	0/11	0	0
16 to 20	20 (3/15)	0/15	12.5 (1/8)	0/8
>21	7.14 (1/14)	14.29(2/14)	0/10	0/10
Sewage				
Sewer	8.2 (5/61)	4.92 (3/61)	5.13 (2/39)	2.56 (1/39)
Septic tank	35.29 (6/17)	5.88 (1/17)	22.22 (2/9)	0/6
Travel history[†]				
Yes	20.83 (5/24)	0/24	0/16	0/16
No	11.11 (6/54)	7.4 (4/54)	12.5 (4/32)	3.13 (1/32)

*Prevalence, individuals with evidence of past WNV or DENV infection.
**Incidence, individuals who contract WNV or DENV infection during May-December 2015
[†]Travel history outside Ciudad Juarez
[#]No. Positive, number of seroconversions to DENV or WNV
[$]No. tested, the total number of samples available

Table 2.4 (Continued). Comparison of dengue and West Nile virus antibody prevalence and incidence of seroconversion among human cohort during May to December 2015 in Ciudad Juarez, Mexico

Variable	**% of Prevalence* (No. positive[#]/ No. tested[$])**		**% of Incidence** (No. positive[#]/ No. tested[$])**	
Age	DENV	WNV	DENV	WNV
18-23	0/10	10 (1/10)	0/8	0/8
24-29	0/6	0/6	40 (2/5)	0/5
30-35	16.67 (2/12)	8.33 (1/12)	0/6	16.67 (1/6)
36-41	10 (1/10)	0/10	0/8	0/8
42-47	21.43 (3/14)	0/14	11.11 (1/9)	0/9
48-53	37.5 (3/8)	12.5 (1/8)	0/4	0/4
54-59	11.11 (1/9)	11.11 (1/9)	0/5	0/5
>60	11.11 (1/9)	0/9	33.33 (1/3)	0/3

*Prevalence, individuals with evidence of past WNV or DENV infection.
**Incidence, individuals who contract WNV or DENV infection during May-December, 2015
[†]Travel history outside Ciudad Juarez
[#]No. Positive, number of seroconversions to DENV or WNV
[$]No. tested, the total number of samples available

2.3. Determine if there is serological evidence of dengue viral infection among a cohort of the pregnant females in El Paso, Texas

2.3.1 Material and methods

2.3.1.1 Study site

El Paso area is located in the western most county of Texas. The estimated population is 678 815 inhabitants (US Census Bureau, 2020), being considered one of the largest cities in Texas. The climate is arid and temperature varies from 2°C to 36°C. Precipitation averages 220mm per year and occurs mainly from June through September being caused by the North American Monsoon. The main economic activities are international trade, military, oil and gas, health care and tourism sectors services.

A serosurvey was performed for DENV and WNV antibodies among pregnant females between 2015 and 2016 at local hospitals in El Paso Texas (**Figure 2.2**), because they were considered appropriate for achieving the aim to determine if DENV were endemic in El Paso community. The study was based on availability of convenient cord-blood samples. The individuals who were asked to participate in this survey were enrolled under a protocol (#515533-7) approved on by the Institutional Review Board (IRB) at the University of Texas at El Paso (UTEP). Furthermore, a similar IRB protocol was approved by the participating hospitals that included Sierra Medical Center (SMC), Sierra East Medical Center (SES) and the Providence Memorial Hospital (PMH), El Paso, TX. All participants in this study were 18 years or older, and all provided a voluntary written informed consent that was witnessed by the study's clinical coordinator. Epidemiological data was collected from the participants including age, sex, zip code/colonia, location, and travel history.

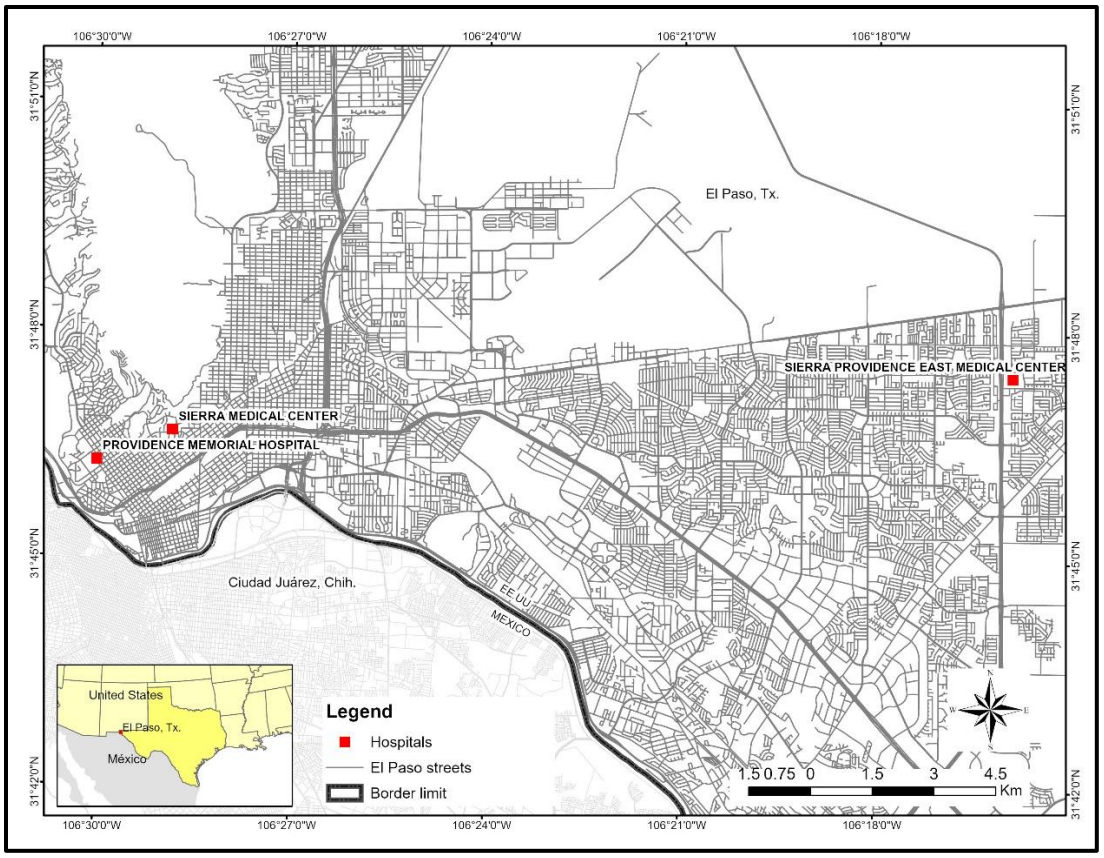


Figure 2.2. Map of El Paso city, Texas, highlighting the location of the hospitals where the blood cord samples were collected**.**

Blood cord samples were collected at the hospitals by the coordinator using standard aseptic techniques with a vacutainer collection tube containing an anticoagulant and then placed in an Igloo container on ice packs, and transported to the laboratory. Samples were centrifuged at 1200 Gs at 4°C for 10 minutes. The plasma samples were transferred to sterile vials and stored at -20°C until tested for IgG and neutralizing antibodies.

2.3.1.2 Indirect Immunoglobulin G (IgG) enzyme-linked immunoabsorbent assay (ELISA)

Plasma samples were tested by an indirect ELISA for IgG antibodies to DENV and WNV as described previously (Section 2.2.1.2).

2.3.1.3 Immunoglobulin M (IgM)-capture ELISA to DENV

Dengue-specific IgM antibody in the blood cord samples was determined by an IgM-capture ELISA as mentioned previously (Section 2.2.1.3).

2.3.1.4 Plaque reduction neutralization test (PRNT)

Plasma samples that were reactive in the indirect IgG ELISA to DENV or/and WNV were tested by PRNT to each of the DENV serotypes: DENV 1 (16007), DENV 2 (16681), DENV 3 (H87), DENV 4 (1036) and WNV (NY99) as described previously (Section 2.2.1.4). Furthermore, neutralization test to Saint Louis Encephalitis virus (SLEV) (TVP 12917) was performed for blood cord samples that were positive for suspected Flavivirus (DENV and/or WNV IgG antibodies positive and DENV/WNV neutralizing antibody negative) following the procedure mentioned above (Section 2.2.1.4) and incubated at 37°C and 5% CO_2 for 5 days. After incubation with any of the above-mentioned viruses, cells were fixed and stained with naphthol blue-black solution. Virus dose was determined as the mean number of PFU (Plaque Forming Unit) recorded on twelve wells cells infected with 30-60 PFU based on testing of an equal volume of a dilution of the virus stock and antibody negative control human plasma. PFUs were counted, and the dilution of plasma that reduced 80% of the virus dose was considered as the antibody titer. Blood cord samples that neutralized both DENV and/or WNV with titers 1:40 or higher were considered antibody positive.

2.3.2. Results

A total of 1,472 pregnant mothers were enrolled in this study and included 562 at the Providence Memorial hospital, 158 at the Sierra Medical Center Main hospital, and 752 at the Sierra Medical East hospital. Most of the mothers (90.83 %, n=1337) were from El Paso, Texas (**Table 2.5**) followed by Mexico (3.74%, n=55) and New Mexico (3.05%, n=45), while some

mothers didn't provide a location (2.3%, n=35). Age distribution revealed that most of the mothers (90.08%) ranged from 18-34 years (33.42 % [n=492] ranged from 18 – 23 years, 38.52 % [n=567] ranged from 24 – 29, and 18.14 % [n=267] ranged from 30 – 34), while other mothers were older than 35 years (7% [n=103] from 35 – 40, 1.15 % [n=17] from 41 – 45, and 0.14 % [n=2] was over 46 years old) or did not state their age 1.63 % (n=24) . Finally, 43.95% of the mothers stated that they travelled outside El Paso in the last 5 years.

A total of 59 samples were positive for DENV and or WNV antibodies (**Table 2.6**). The seroprevalence rate for WNV and DENV only was 3.06% (n=45) and 0.54% (n=8) respectively, being 0.2% (n=3), the rate for both DENV and WNV antibody**.** As a consequence, the overall seroprevalence rate for WNV and DENV, considering the DENV/WNV positives, was 3.26% (n=48) and 0.74% (n=11) respectively. Three samples (0.2%) were considered as suspected Flavivirus due were positive for IgG antibody for both DENV and WNV only. The distribution of WNV antibody positive rate was 1.78%, 4.43% and 3.72% in the Providence Memorial, Sierra Medical Center, and Sierra Medical East hospital, respectively. In case of DENV antibody, 1.27% and 0.8% positive rates were from the Providence Memorial and Sierra Medical East hospitals respectively. WNV and DENV antibody positives represented the 0.36% of the samples from Providence Memorial hospital.

Of the 56 samples that were positive for DENV and/ or WNV neutralizing antibody by $PRNT_{80}$ (**Table 2.7**), 45 samples had a monotypic WNV antibody, 3 samples had a monotypic DENV antibody (1 sample had DENV 1 and 2 samples had DENV 2 neutralizing antibodies only), 5 samples had a polytypic DENV antibodies (3 samples had DENV 1/DENV 2 neutralizing antibodies and 2 samples had DENV 1/DENV 2/DENV 3 neutralizing antibodies),

and 3 samples had polytypic DENV and WNV antibodies (2 samples had DENV 2/WNV antibodies and 1 sample had DENV 3/DENV 4/WNV antibodies).

Table 2.5. Description of the participant pregnant mothers enrolled from El Paso, Texas

Sociodemographic characteristics	**N (%)**
Location	
El Paso	1337 (90.83%)
Mexico	55 (3.74%)
New Mexico	45 (3.05%)
No information	35 (2.38%)
Age (years)	
18-23	492 (33.42%)
24-29	567 (38.52%)
30-34	267 (18.14%)
35-40	103 (7%)
41-45	17 (1.15%)
>46	2 (0.14%)
No information	24 (1.63%)
Travel history	
Yes	647 (43.95%)
No	807 (54.83%)
No information	18 (1.22%)

Table 2.6. Seroprevalence of Dengue and West Nile virus antibody among pregnant woman from El Paso, Texas

	Providence Memorial	**Sierra Medical Center**	**Sierra Medical East**	**Seroprevalence rate**
	no. (%)	no. (%)	no. (%)	no. (%)
West Nile	10 (1.78 %)	7 (4.43 %)	28 (3.72 %)	45 (3.06%)
Dengue	0	2 (1.27%)	6 (0.8 %)	8 (0.54 %)
Dengue/West Nile	2 (0.36%)	0	1 (0.13 %)	3 (0.2 %)
Suspected Flavivirus	0	2 (1.27%)	1 (0 .13)	3 (0.2%)
Total	12/562 (2.14%)	11/158 (6.96%)	36/752 (4.78%)	59/1,472(4.0%)

Table 2.7. Neutralizing antibody to Dengue and West Nile virus infection among pregnant woman from El Paso, Texas

Virus	**Providence Memorial** (n =562)	**Sierra Medical Center** (n =158)	**Sierra Medical East** (n=752)	Total (n=1472)
DENV-1			1 (0.13%)	1 (0.07%)
DENV2		1 (0.63%)	1 (0.13%)	2 (0.14%)
DENV1/DENV2		1 (0.63%)	2 (0.26%)	3 (0.20%)
DENV1/DENV2/DENV3			2 (0.26%)	2 (0.14%)
DENV2/ WNV	2 (0.36%)			2 (0.14%)
DENV3/DENV4/WNV			1 (0.13%)	1 (0.07%)
WNV	10 (1.78%)	7 (4.43%)	28 (3.72%)	45 (3.06%)
No antibody	550 (97.86%)	149 (94.3%)	717 (95.35%)	1417 (96.2%)

2.4. Discussion

This study represents the first detection of serological evidence of DENV in humans in the El Paso Texas-USA and Ciudad Juarez-Mx border region. Previous surveillance of human DENV infection along the US-Mx border has been performed occasionally in certain DENV endemic areas in Brownsville, Texas and Matamoros, MX and surrounding urban communities in the Rio Grande Valley of Texas after sporadic dengue outbreaks (Brunkard et al., 2007; Reiter et al., 2003; Thomas et al., 2016).

Our results indicated that the evidence of past DENV infection in the Ciudad Juarez and El Paso communities was 14.1% and 0.74% of the study participants, respectively. However, the 14.1% and 0.74% DENV seroprevalence rate for past infection in Ciudad Juarez and El Paso during this study was lower than the 78% rates reported during 2004 in the residents of Matamoros, Mexico and 40 % rate reported in Brownsville, Texas (Brunkard et al., 2007). Furthermore, the seroprevalence rate for past infection in the Ciudad Juarez / El Paso community was lower than the 48% in Nuevo Laredo, Mexico and 23% rate reported for Laredo, Texas(Reiter et al., 2003). Overall, the seroprevalence rates of DENV infections have been reported to vary in the border communities. Hotez, 2008 estimated that more than 100 000 dengue infections occur annually among the entire border population of 10 million people (Pew Research Center, 2015), based on the observation that 2% of humans in Brownsville had a recent DENV infection (Brunkard et al., 2007).

Out of the 11 DENV antibody positive cord-blood plasma samples from El Paso, 7 samples were from the Sierra Medical East, 2 from the Providence Memorial, and 2 from Sierra Medical Center hospitals. Mothers with DENV antibodies resided mainly in the East El Paso (n=8), where the density of *Ae. aegypti* mosquitoes was higher during the summer/fall from

2015-2017 (Watts D.M., unpublished data). Furthermore, *Ae. aegypti* is considered the second most abundant mosquito species in El Paso. On the other hand, it can be a possibility that the pregnant mothers might have been exposed to DENV outside El Paso, as 5/11 mothers (45.5%) indicated previous travel to DENV endemic countries as Mexico and Puerto Rico. In case of Ciudad Juarez, a total of 11 DENV antibody positives were detected in the first serosurvey where almost 50% of the positives (5/11), stated previous travel outside Ciudad Juarez. Both El Paso and Ciudad Juarez (first survey) samples were tested for IgM as evidence of recent infection. Our result indicated that all the DENV positive blood cord and sera samples from El Paso and Ciudad Juarez (first survey) respectively were negative for IgM antibody. In case of the blood cord samples, these results matched the knowledge that IgM antibody rarely cross the placental barrier while IgG antibodies to DENV were reported to be transferred across the placental barrier in several DENV hyperendemic countries as in Thailand, Brazil with a rate of 96.8% and 99.3%, respectively (Argolo et al., 2013; Watanaveeradej et al., 2003).

The overall DENV seroprevalence rate obtained from El Paso might represent the rate among a male cohort. Previous studies in Mexico and Peru have indicated that dengue cases were similar among males and females, or more common among females than males (Forshey et al., 2010; Rojas et al., 2018). Furthermore, the low DENV seroprevalence in El Paso differs from other areas with DENV endemic transmission in the Rio Grande Valley, suggesting that it did not reflect autochthonous DENV transmission. Even though, active surveillance for DENV has not been conducted in El Paso, no DENV were isolated from *Ae. aegypti* during 2015-2017 collected in the East, Central and West El Paso area (Watts D.M., unpublished data), supporting the lack of autochthonous DENV transmission in this border community.

In case of the serological surveillance in Ciudad Juarez, our results represent the first evidence of endemic DENV transmission along the U.S. – MX border west of the Rio Grande Valley of Texas. Seroconversions in our study as evidence of a recent DENV infection was detected in 8.3% (4/48) of the study participants. Although the sample size was much lower in our study, the rate of recent infections was higher than the 2% and 7.3% rates reported during 2004 for residents of Brownsville and Matamoros, respectively (Brunkard et al., 2007). In Nuevo Laredo, the rate of recent DENV infection during an outbreak, in 1999 was 16%, but in Laredo, Texas, the rate of 1.3% was substantially lower than our finding of 8.3% rate in Ciudad Juarez (Reiter et al., 2003).

Our findings are the first evidence of DENV infections in humans in Ciudad Juarez, MX, where dengue RNA was detected during 2005 in field-collected *Ae. aegypti* mosquitoes (de la Mora-Covarrubias et al., 2010). In addition, even though the sample size of this study was small (n = 78), the serological data supported the local circulation of DENV in Ciudad Juarez because of the detection of neutralizing antibodies to DENV 1 and DENV 2 serotypes and DENV IgM antibody in 2 of 4 study participants who seroconverted to DENV infection. Also, the individuals with DENV seroconversion did not report any travel history outside Ciudad Juarez, and 3 of the 4 individuals stated that most of their time was spent at home during the survey, suggesting that DENV infection occurred at home (**Table 2.4**). However, further studies are needed to confirm this possibility. An understanding of our observations for 2 of the participants who were negative during the first survey for DENV antibody but were positive for DENV IgG antibody and negative for DENV IgM antibody during the second survey is unknown. However, it is possible that the IgM antibody waned to undetectable levels for these 2 individuals during the 5-6 months interval between the initial and follow-up survey period.

As an example of the possible underestimation of dengue cases, the RGV is the better-known region of endemic DENV transmission on both sides of the border. Still, the understanding of the ecology and epidemiology is insufficient because of the lack of any systematic and poorly designed studies that have failed to provide an accurate estimate of the incidence and the public health impact of the DENV infections. As an example, during 1980-1999, there were 65,514 cases of dengue reported from the MX side of the border in the RGV as compared to only 64 cases on the U.S. side of the border (Brunkard et al., 2007; Gubler et al., 2001; Reiter, 2001). One study suggested that this disparity could be attributed to human behavior and the air conditioning system on the U.S. side of the border (Reiter et al., 2003). However, a subsequent study revealed that dengue cases were being under-reported in the U.S. near the Mexican border (Brunkard et al., 2007). Efforts to understand possible reasons for the underestimation of cases indicated that this could be attributed to passive surveillance (Hafkin et al., 1982). For example, during the 1980 outbreak in Texas, passive surveillance failed to detect any dengue cases, but 63 cases were detected by active surveillance at out-patient clinics, including 52 (83%) in counties adjacent to the Texas-MX border. Another example related to misdiagnosis of cases was during a dengue outbreak in 2013 in Brownsville when less than half of the cases were reported to the Texas Department of State Health Services because commercial laboratory diagnostic testing results were false negative for about one in 5 of the cases (Thomas et al., 2016). Other possible reasons were that dengue cases might go unrecognized is because DENV strains caused mostly silent or subclinical infections and mild diseases. Also, 59% of Brownsville residents cross the border into MX for medical care which is likely to limit disease reporting on the U.S. side of the border (Brunkard et al., 2007; C. for D. C. and P. (CDC, 2001).

In this study, serological evidence of past (5.13%, n =4) and recent (n = 1 seroconversion) WNV infections were detected in humans in Ciudad Juarez, where WNV positive *Cx. quinquefasciatus* mosquitoes were reported in a previous study (Mann et al., 2013). Meanwhile in El Paso, the WNV seroprevalence was 3.26%, where a total of 312 cases of West Nile has been reported since 2003 and WNV has been isolated from *Culex* mosquitoes (TDSHS, 2021). A similar low WNV seroprevalence (4%) was reported in Colorado from cord blood samples (Paisley et al., 2006). Our results are comparable to other studies in North America/Canada and Europe/Middle East where the WNV seroprevalence ranged from 2.65 to 14% and 2.1% to 11.1% respectively, while in Africa, the WNV rates ranged from 2.75% to 59% (Biçeroğlu et al., 2015; Mostashari et al., 2001; Murphy et al., 2005; Nagy et al., 2022; Nur et al., 1999; W. Wang et al., 2009). Overall, these results support WNV transmission in Ciudad Juarez and El Paso communities as previously reported in El Paso (Cardenas et al., 2011) and northern MX (Nuevo Leon) (Rodríguez et al., 2010).

Since our testing was for only DENV and WNV antibodies, we cannot exclude the possibility that other *Flaviviruses* were circulating in Ciudad Juarez and El Paso such as St Louis encephalitis virus or ZIKV because of the possibility of cross-reactivity that has been reported among Flaviviruses, especially among humans who had secondary infections (Lanciotti et al., 2008). However, ZIKV has not been reported from the Ciudad Juarez during the study but has been documented in other areas, especially in Chiapas, MX (Guerbois et al., 2016). In addition, the distribution of neutralizing antibodies to DENV and WNV in the 12 individuals with paired samples from Ciudad Juarez were similar for the baseline (May-June 2015) and the second survey (November-December 2015), suggesting that ZIKV and/or other Flaviviruses infection were not involved in the neutralizing antibody response in those 12 individuals. In case of El

Paso study, only 3 samples were negative for WNV or DENV neutralizing antibodies but reactive for *Flaviviruses* IgG antibody and possibly attributed to the cross-reacting antibodies for other *Flaviviruses* or non-specific reactivity.

Our results suggested the endemic transmission of DENV in Ciudad Juarez and that further studies are warranted to determine the incidence and clinical outcome of DENV infections in the Ciudad Juarez community. This observation is further supported by a recent report of 1 and 9 dengue cases in this community during 2016 and 2017, respectively (DGE, 2016, 2017), and therefore, is likely to reflect an increasing trend in dengue cases. In contrast, DENV is not likely to be endemic in the El Paso community due to the very low DENV seroprevalence (0.74%) in comparison with other endemic DENV areas (>67%) (Carabali et al., 2017; Morrison et al., 2010; Vongpunsawad et al., 2017).

In case of WNV, the lack of WN cases reported in Ciudad Juarez and other Mexican areas could be related to the limited resources for testing human samples, or else the endemic level of WNV is low. Unlike Ciudad Juarez, El Paso community have reported WN human cases since 2003 and mosquito surveillance by the City of El Paso Surveillance and Vector Control Program and the Texas State Health Services and research institutions have detected WNV infected *Culex* mosquitoes. However, further studies are needed to determine the clinical outcome of WN in the Ciudad Juarez and El Paso community.

Finally, longitudinal cohort studies and mosquito surveillance could lead to a better understanding of the transmission dynamics and public health importance of DENV and WNV in this border community and provide critically needed data to develop more effective mosquito control programs.

Chapter 3: Vector Competence Of *Ae. Aegypti* Mosquitoes From The U.S. Border Region For Dengue 2 Virus And The Effect Of Viral Dose And Temperature

3.1 Introduction

Aedes aegypti mosquitoes are distributed along the United States (U.S.) border region. Ae aegypti is considered the main vector of dengue virus (DENV) because its feature to replicate and transmit the virus to humans, feature also known as vector competence. Now, even though *Ae. aegypti* mosquitoes have been well documented as the primary vector of DENV, there is limited evidence of the vector competence of this species that inhabit the southern regions of the U.S. Previous vector competence studies involving DENV and *Ae. aegypti* from Mexico and the U.S. (Tucson and Houston), indicated variation in vector competence (Bennett et al., 2002). Interestingly, two populations from the Mexico border communities (Nuevo Laredo and Tamaulipas) displayed moderate vector competence.

Mosquito vector competence is associated with some specific combinations of the genetic of the mosquito vector and genetic characteristics of the virus. This genotype-by-genotype interaction has been determined in *Ae. aegypti* and *Ae. albopictus* mosquito populations with different Chikungunya virus (CHIKV) strains, causing different viral transmission efficiencies (Tsetsarkin et al., 2007) . This information suggested the potential risk of transmission of some viral strains in an area with the presence of a mosquito's species. In addition, *Ae. aegypti* and *Ae. albopictus* from Florida were found to be highly susceptible to an American strain of DENV-1, but with a low transmission rate (Richards et al. 2012). Currently, there is a lack of information on whether *Ae. aegypti* from the U.S. border are susceptible to certain DENV serotypes and strains.

Temperature as an environmental factor tends to induce variations in the vector efficiency of *Ae.aegypti* to transmit DENV (Watts et al., 1986). However, to date, there is no data on the effects of temperature on the vector competence of *Ae. aegypti* mosquitoes from U.S. border

communities. Experimental and field studies could help to understand the effect of temperature in temperate areas where transmission of these arboviruses is minimal, especially the possibility of increasing temperature due to global warming. The EIP dependence on temperature needs to be addressed as new emerging viral diseases continue to present a threat to human and animal health in the Americas. As described in this chapter, experimental studies were conducted to evaluate the vector competence of *Ae. aegypti* for DENV serotype 2, including the effect of temperature and viral dose on virus transmission. The specific aims are as follows:

Aim 1: Evaluate the susceptibility of *Ae. aegypti* from El Paso and Rio Grande to DENV-2.

Aim 2: Determine the vector competence of Ae. aegypti from the U.S. border region for different strains of DENV-2 based on infection rates, distribution of virus in the body, wings and legs of the mosquitoes and ability to transmit the virus

Aim 3: Evaluate the effect of a lower DENV-2 viral dose in the vector competence of U.S. border region *Ae. aegypti*.

Aim 4: Evaluate the effect of temperature on the vector competence of U.S. border region *Ae. aegypti.*

3.2. Evaluate the susceptibility of *Ae. aegypti* mosquitoes from El Paso and Rio Grande to dengue 2 virus

3.2.1. Material and Methods

3.2.1.1 Standard rearing techniques for *Ae. aegypti*

The term "strain" was used to refer to *Ae. aegypti* collected from different sites along the U.S – Mexico border communities. Briefly, 2 strains of *Ae. aegypti* were reared in the UTEP insectary (Biology Building, Room 207), maintained at 28ºC, 70% relative humidity, and 14h: 10h light: dark cycle (**Table 3.1**). Briefly, mosquito eggs were collected in the field from two U.S. border communities (El Paso and Brownsville counties) and hatched in deoxygenated and deionized water containing 0.5% yeast. First instar larvae were distributed 400 per container in 1 litter of water and fed with 0.4 mg of Tetramin flakes fish food (Tetra, Blacksburg, VA) /larvae. Second instar larvae were fed with 0.6 mg Tetramin. Third instar larvae were fed with 0.8 mg and 4^{th} instar larvae were fed with 1-2 mg/larvae. Pupae were transferred to a 30 cm^3 mosquito holding cage and a 10% cotton ball saturated with a sugar solution was provided ad libitum. After emergence, adult mosquitoes were cold-anesthetized and identified using a dichotomous taxonomical key (Darsie et al., 2005) . Once identified, four days later, *Ae. aegypti* mosquitoes were blood-fed with defibrinated sheep blood (Colorado Serum Company, Denver, CO) using a glass feeder or an Hemotek feeding system **(**Hemotek Ltd, Blackburn, UK) covered with a desalted pig casing membrane. Two to three days after feeding, an oviposition cup containing a piece of germination paper partially covered with water was placed inside the mosquito cage to allow the mosquitoes to oviposit, and for collecting eggs. Three days later, the paper containing the mosquito eggs (known as egg paper) was removed and stored in sealed bags at 28ºC and 80% relative humidity. Sheep blood was offered to the adult female mosquitoes every week and the

eggs were collected as mentioned above. Successive mosquito generations were maintained as described above and the third generation of mosquitoes was used for the vector competence experiments to be more similar to the natural populations of *Ae. aegypti.*

Table 3.1. Strain history of *Ae. aegypti* mosquitoes used for this study

Strain	Collection Data
El Paso Northeast, TX	Eggs from ovitraps in El Paso, Northeast area, Texas, were collected in June-September 2018 by the UTEP Mosquito Ecology Surveillance Laboratory.
Rio Grande TX	Eggs from ovitraps in Brownsville, Texas, collected in March-April 2018 by Dr. Christopher Vitek, a professor in the Department of Biology at the University of Texas Rio Grande Valley, Edinburg, Texas.

3.2.1.2. Viruses

Dengue virus serotype 2 (DENV-2), including 3 genotypes (Asian, American, and Southeast Asian/American) were used to infect C6/36 cells (*Ae. albopictus* cell line) grown in T-25 cm^2 flask for producing viral working stocks using a DENV pool method **(Table 3.2)**. Briefly, confluent monolayers of C6/36 cells were infected with DENV-2 strains at a multiplicity of infection (MOI) of 0.01, and 5 mL of maintenance medium (Eagle medium with 2% fetal Bovine Serum [EMEM- 2% FBS]) was added to each flask. Two days post-infection (p.i.); the media was removed and replaced with 5 mL of EMEM without FBS. At day 4 p.i., the supernatant medium was collected and stored at 4ºC (1st collection) and replaced with fresh EMEM without FBS. The same procedure was performed on day 6 p.i. (2nd collection); and the last collection (3rd collection) was done on day 8 p.i. Evidence of viral cytopathic effect (CPE) in the cells was monitored daily using an inverted microscope. Non-infected C6/36 cells were used as controls and tested using the same procedure. After the incubation period, C6/36 cells were scraped off the flasks and the supernatants were clarified by centrifugation at 4,000 g for 10 minutes at 4ºC.

Supernatants were stored at -80°C until tested to determine the DENV-2 infectivity titers. Cellular pellets were resuspended in Phosphate Buffer Saline (PBS) 1X, spotted onto the ringed area of ring slides, dried, and fixed in cold acetone at -20°C for 10 minutes to screen for DENV-2 using the indirect fluorescence antibody assay.

The infectivity titers of the DENV-2 stocks in the supernatant of infected C6/36 cells were quantified by plaque assays (plaque-forming units/mL [PFU/mL]) in baby hamster kidney 21 cells (BHK-21). If the pooled supernatants were positive for DENV-2, 0.5mL aliquots each were transferred to sterile 2.0 ml vials and stored at -80ºC and considered as the viral stock. Viral stocks for each DENV-2 strain was prepared at different times to avoid cross-contamination. The stocks of DENV-2 were used in experiments to test mosquitoes for susceptibility to DENV-2 infection by using freshly harvested DENV-2. Furthermore, prior to the experimental mosquito infections with DENV-2, viral replication curves were performed to determine the optimal time for harvesting the viral suspension for providing mosquitoes the desired infectious dose of the virus (**Appendix 1**).

Table 3.2. DENV-2 strains used to evaluate the vector competence of Ae. Aegypti

Strain of DENV- 2	**Source of DENV- 2 Isolate**	**Country**	**Passage history**	**Genotype**	**Source (Providers)**
FSB 3381	Human	Bolivia	C6/36#3	Asian-American	World Reference Center for Emerging Viruses and Arboviruses - University of Texas Medical Branch, Galveston, Texas
328298	Human	Mexico	C6/36#5	American	Department of Pediatrics -Baylor College of Medicine, Houston, Texas
Prototype 16681	Human	Thailand	C6/36#9	Asian	Naval Medical Research Center Unit 6, Lima, Peru

3.2.1.3. Viral Identity assays

3.2.1.3. 1. Dengue viral Sequencing

Sequencing of the envelope gene from each DENV-2 strain was performed to authenticate DENV-2 genotypes (**Table 3.2**). Briefly, DENV-2 RNAs were extracted using the QIAamp viral RNA kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Pre-Membrane (preM) and envelope (E) genes of the DENV-2 strains were amplified by a reverse transcription polymerase chain reaction (RT-PCR) for further phylogenetic analysis (Warrilow et al., 2012). Amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and sequenced using the BigDye® Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), with the sequencer 3730xl DNA analyzer (Applied Biosystems). Envelope gene sequences were analyzed and edited with Sequencher 4.6 (Gene Codes Corporation, Ann Arbor, MI) and compared with other sequences deposited in the

GenBank database using the BLASTn software. Sequences were analyzed by using the neighbor-joining and the maximum probability analysis implemented in PAUP (test version 4.0b 10). The best model was determined by Mr. Model test software. Phylogenetic clades of DENV-2 genotypes were evaluated by bootstrap analyses with 1000 replicates for maximum probability analyses.

3.2.1.3.2. Immunofluorescence assay test (IFAT)

IFAT was used to confirm the identity of the DENV-2 stocks. IFAT was performed by using polyclonal antibodies ascites to Saint Louis encephalitis virus (SLEV) which is cross-reactive to DENV antigen; and polyclonal and monoclonal antibodies ascites againstDENV-1 and DENV-2. Briefly, 10 µL of polyclonal or monoclonal antibodies were added onto the ringed circles of slides and incubated 37ºC for 1 hour. Then, slides were washed twice in PBS 1X, dried and 10 µL of mice antibody conjugated with Fluorescein Isothiocyanate 1:100 diluted in 1x PBS were added and incubated at 37ºC for 1 hour. DAPI (4',6-diamidino-2-phenylindole) was used as the counterstain (1:1000 dilution). Slides were washed twice in PBS 1X, dried, and a drop of a glycerol solution (9 parts glycerol + 1 part PBS) was added onto the slide. Fluorescence as evidence of the virus identity was detected by using a 20x objective on a fluorescent microscope (Nikon, Ti-S). Slide with controls containing infected or uninfected cells were subjected to the same procedure.

3.2.1.4 Oral mosquito infections

The third (F3) *Ae. aegypti* generations were tested for oral infection with DENV-2 (FSB 3381) and DENV-2 (328298) strains (**Table 3.1**). Two days before each experiment, female adult mosquitoes were aspirated from the cages and placed in 16 ounces paper cup cages for acclimatization. The next day, 10% sugar solution source was removed and replaced with water.

On the day of infection, four to five days-old female mosquitoes were offered an infectious blood-meal consisting of 2 mL defibrinated sheep blood (Colorado Serum Company, Denver, CO), combined with 1 mL of freshly propagated DENV-2 suspension, harvested on day 4 p.i., and offered in 3 mL membrane covered feeder attached to a heated feeding system (Hemotek Ltd, Blackburn, UK) kept at 37ºC and covered with a mouse skin membrane. Artificial virus-blood meals were offered to the mosquitoes for 45 minutes. An aliquot of the infectious blood meal was collected before and after feeding the mosquitoes to determine the infectivity titer of DENV-2 in the blood meal ingested by the mosquitoes.

After mosquitoes ingested the mixture of DENV-2 and blood, mosquitoes were cold anesthetized, and a group of only fully engorged females were transferred to 16-ounce mesh-top cardboard cups and fed with 10% sucrose in a chamber maintained at standard temperature (28°C, at 14h: 10h light: dark cycle and 80% humidity for 7 and 14 days. In order to determine the amount of DENV-2 ingested by the mosquitoes, immediately after ingesting the virus-blood mixture, two to five engorged females were stored at -80°C until tested individually to determine the infectious virus dose by plaque assay in BHK-21 cells. At days 7 and 14 dpi (days post infection), batches of individual mosquitoes were removed from the holding cages and stored at -80°C until tested for viral infection.

3.2.1.5. Mosquito processing

Individual mosquitoes were tested to estimate DENV infectivity rates for each *Ae. aegypti* mosquito strain. Briefly, individual mosquitoes were homogenized in 500 µL of media (EMEM 2% FBS maintenance medium supplemented with 1% amphotericin B, and 2 % Penicillin/Streptomycin) using a TissueLyzer II (Qiagen, Valencia, CA) for 4 minutes at 30 cycles/second containing a 3 mm tungsten bead per mosquito. Homogenates of mosquitoes were

clarified by centrifugation at 10,000 g for 10 min at 4ºC and the supernatants were tested to determine viral infectivity and titers by plaque assays in C6/36 and BHK-21 cells.

3.2.1.6. Viral detection and quantification

3.2.1.6.1. Viral isolation

One hundred microliters of the clarified mosquito suspensions were inoculated onto 96 well- plates containing C6/36 cells and kept for 7-10 days at 28ºC and 5% CO_2 and monitored once each day for CPE. After incubation, the cells were scraped off the plate and the supernatants were clarified by centrifugation and stored at -80°C. The C6/36 cell pellets were spotted onto ring slides and fixed in cold acetone at -20°C and used to perform the IFAT to determine viral infection rates for the mosquitoes.

3.2.1.6.2. Immunofluorescence assay test (IFAT) to DENV

IFAT was used to confirm if the C6/36 cells inoculated with the mosquito tissue suspension were infected with DENV-2. IFAT was performed as described previously (Section 3.2.1.3.2) but using polyclonal and monoclonal antibodies ascites (3H5) against DENV-2.

3.2.1.6.3. Plaque assays

The infectivity titers of dengue viral stocks and clarified mosquito supernatants were determined by plaque assay using a suspension of BHK-21 cells as previously reported (Morens et al., 1985). Briefly, fifty microliters of the tenfold dilutions of the viral stocks or mosquito sample was inoculated onto duplicates suspensions of $3x10^5$ BHK-21 cells/ mL grown in 24-well plates. After 3 hours of incubation at 37ºC and 5% CO_2, the cells were overlaid with a semisolid carboxymethylcellulose 1% overlay and returned to the incubator for 6 days. On day 6, the overlay was removed, and the cells were stained with a naphthol black solution to reveal any

PFU. The PFUs were counted and the viral titers of each mosquito suspension or the dengue viral stock was expressed as PFU/mL.

3.2.1.7. Statistical Analysis

Infectivity rates were calculated as a percentage based on the proportion of the virus-positive mosquito bodies of the total number of mosquitoes fully engorged after the infectious blood feeding. A Fisher exact test was performed using GraphPad Prism version 8.0 and was used to determine significant differences between the infectivity rates in the *Ae. aegypti* mosquito strains and DENV-2 and the incubation period.

3.2.2. Results

3.2.2.1 Sequencing of DENV-2 strains and viral antigenicity

DENV-2 viral stocks antigenicity was confirmed by using DENV-2 monoclonal antibodies (**Figure 3.1**). Phylogenetic analysis of the E gene of the DENV-2 stocks is represented in the **Figure 3.2**. In the case of the DENV-2 (FSB 3381), this strain grouped with other DENV-2 strains that belongs to the Asian/American genotype. Furthermore, DENV-2 (328298) and DENV-2 (16681) strains grouped with the American and Asian 1 genotypes, respectively (**Figure 3.2**).

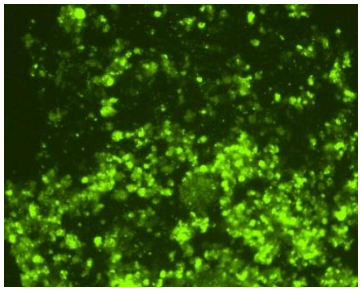
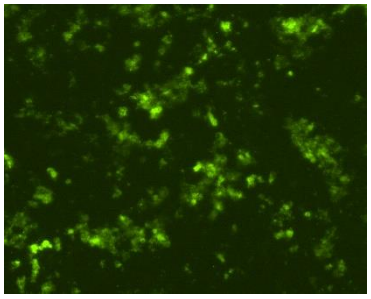
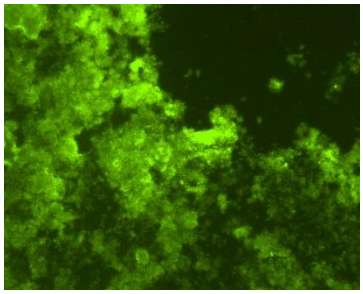
DENV-2 (FSB 3381)	**DENV-2 (328298)**	**DENV-2 (16681)**
		

Figure 3.1. Indirect immunofluorescent antibody assay in C6/36 cells (200X magnification). C6/36 cells were infected with DENV-2 (FSB 3381), DENV-2 (328298), or DENV-2 (16681 strains). Viral antigen was detected in the cells by using the 3H5 monoclonal antibody to DENV-2.

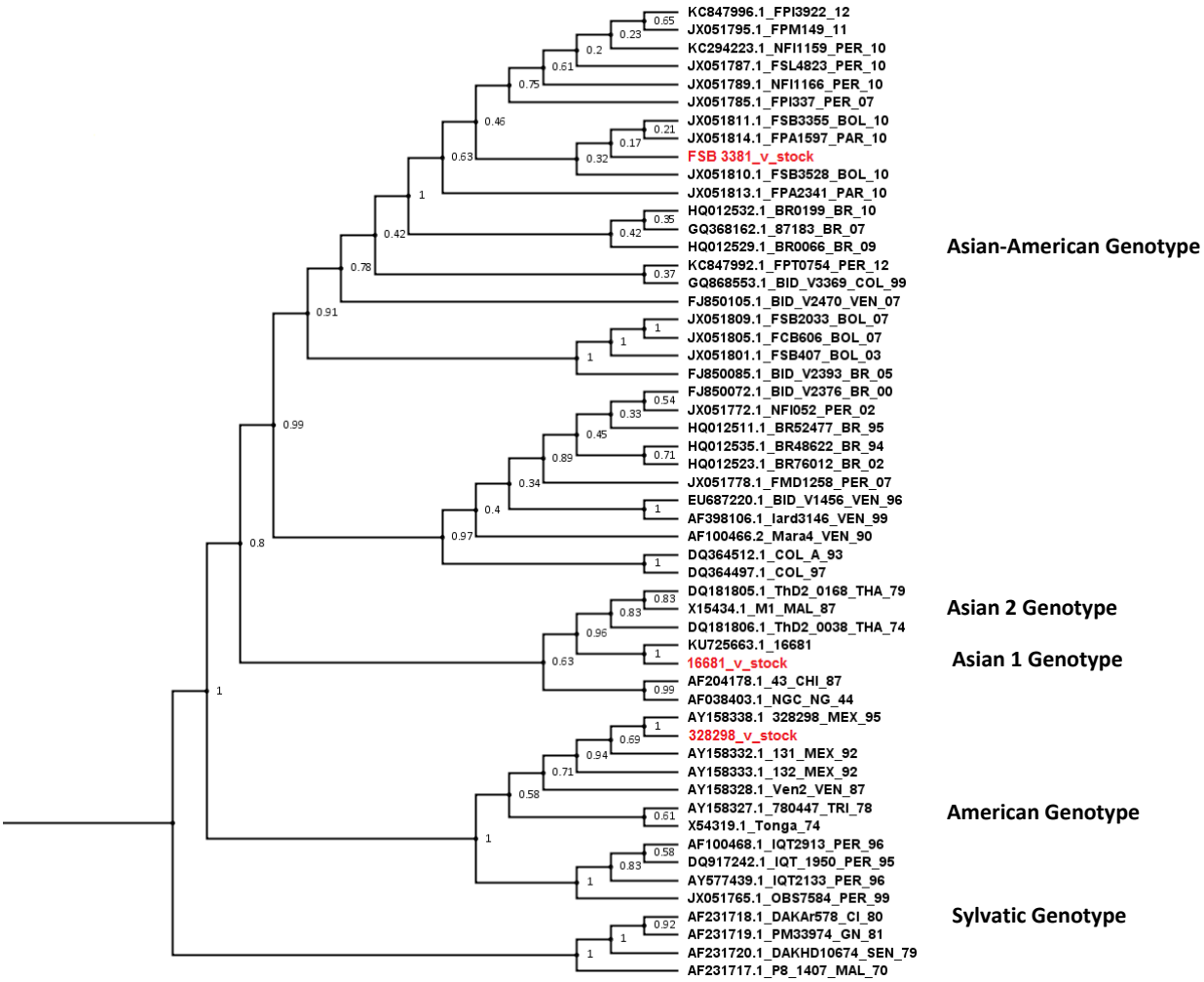


Figure 3.2. Molecular phylogenetic analysis by Maximum Likelihood method derived from 54 DENV-2 envelope glycoprotein gene sequences. Most of the DENV-2 genotypes and isolates are shown by using brackets. Red highlighted legends represented the DENV-2 stocks used in this study. The tree was rooted with sylvatic DENV-2 strains.

3.2.2.2 El Paso *Ae. Aegypti* mosquitoes are susceptible to DENV-2

A total of 41 El Paso *Ae. Aegypti* mosquitoes ingested 5.5 log $_{10}$ PFU/mL of the DENV-2 Asian/American genotype (FSB 3381) and maintained at 28°C and 80% RH for 7 and 14 days. The infectivity rate represented the percentage of virus positive whole mosquito's suspensions of the total blood-fed mosquitoes that were able to infect either BHK and/or C6/36 cell lines and were further detected by plaque or immunofluorescence assays respectively (**Figure 3.3**). El Paso mosquitoes were susceptible to DENV-2 (FSB 3381) infection at 7 and14 dpi, displaying an infectivity rate of 71.43% (15/21) and 75% (15/20), respectively (**Figure 3.4**). The infectivity rate for mosquitoes did not differ significantly between incubation days 7 and 14 ($p > 0.999$).

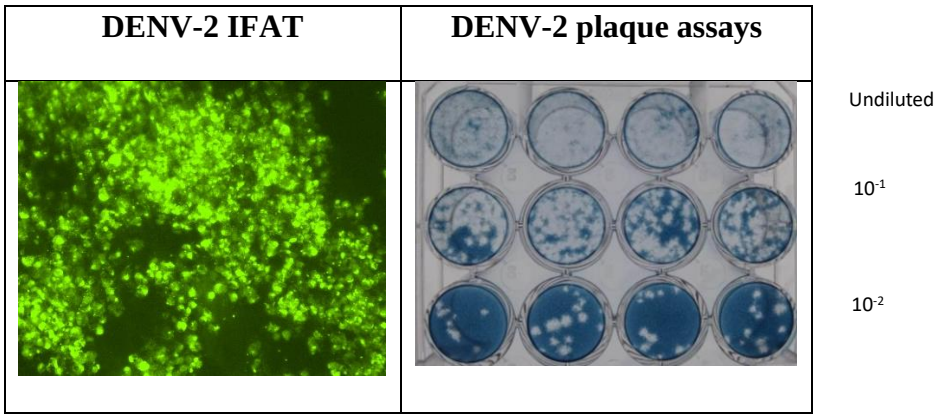


Figure 3.3. El Paso *Ae aegypti* mosquitoes that ingested 5.5 log $_{10}$ DENV-2 (FSB 3381) were ground and suspensions were subjected to IFAT and plaque assays to DENV. DENV-2 viral antigen and titer are shown in (**A**) IFAT in C6/36 cells (200X magnification) and (**B**) plaque assays showing plaque forming units in BHK-21 cells respectively.

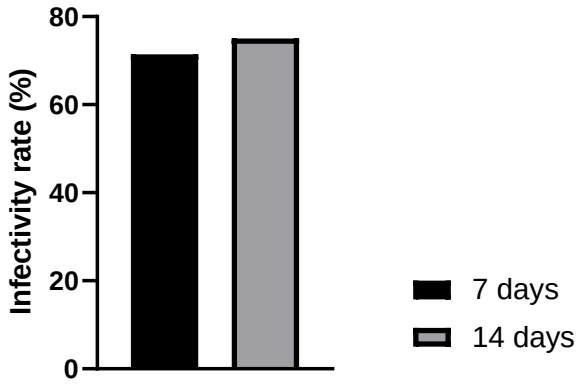


Figure 3.4. Infectivity rate of El Paso *Ae. aegypti* on days 7 and 14 dpi. after ingesting DENV-2 (FSB 3381) in a mixture of blood and virus containing 5.5 $\log_{10}$ PFU/ mL

Ae. aegypti mosquitoes from El Paso were tested for susceptibility to infection with another DENV genotype, or DENV-2 American genotype (328298) using a similar viral dose and incubation period. Overall, the infectivity rates at 7 and 14 days were 91.67% (22/24) and 93% (40/43), respectively (**Figure 3.5**). As occurred with the DENV-2 (FSB 3381), no association was found between the 7- and 14-day incubation period and the infectivity rates ($p>0.05$).

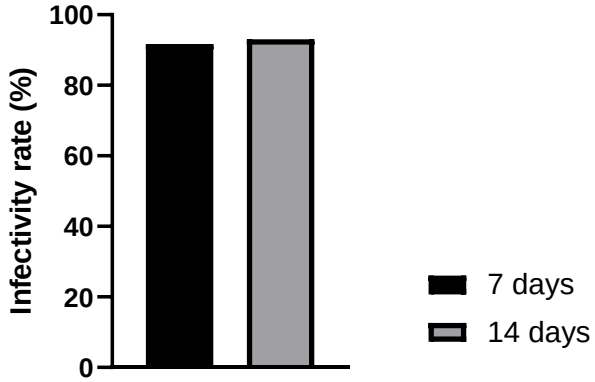


Figure 3.5. Infectivity rate of El Paso *Ae. aegypti* after ingesting DENV-2 (328298) in a mixture of blood and virus containing 5.5 $\log_{10}$ PFU/ mL at 7 and 14 dpi.

Finally, even though a slightly higher DENV-2 (328298 strain) infectivity rate for El Paso mosquitoes was observed, no significant association was observed between the two DENV-2 viral strains and the infectivity rates at 7 and 14 dpi. ($p>0.05$) (**Figure 3.6**).

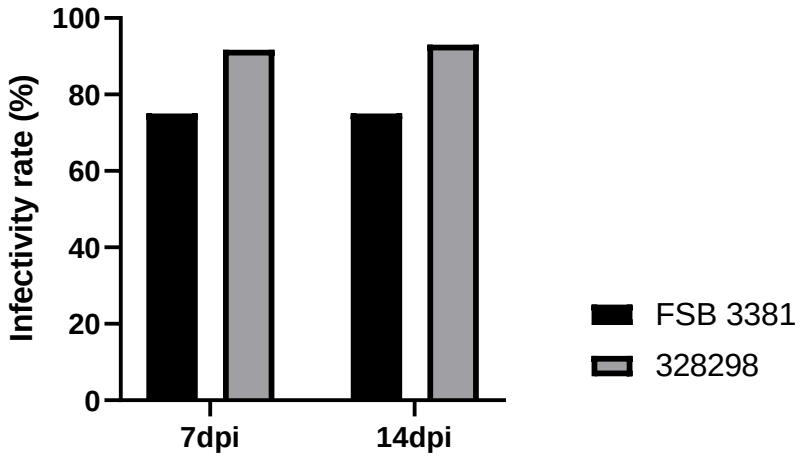


Figure 3.6. Summary of the infectivity rate of El Paso *Ae. aegypti* after ingesting a blood meal containing of 5.5 $\log_{10}$ PFU/ mL of the DENV-2 (FSB 3381) and DENV-2 (328298) at 7 and 14 dpi.

3.2.2.3. Rio Grande *Ae. aegypti* mosquitoes are susceptible to DENV-2

Another U.S. border *Ae. aegypti* mosquitoes (Rio Grande) were tested to determine their DENV-2 susceptibility to infection with DENV-2. The mosquitoes were allowed to ingest 5.5 $\log_{10}$ PFU/ mL of DENV-2 (FSB 3381) and DENV-2 (328298) and maintained at 28°C for 7 and 14 days. On testing these mosquitoes, the infection rate was 80% (8/10) and 84% (11/13) at 7 and 14 dpi, respectively after exposure to the DENV 2 (FSB 3381) (**Figure 3.7**). No association was observed between incubation period and infectivity rate ($p>0.05$).

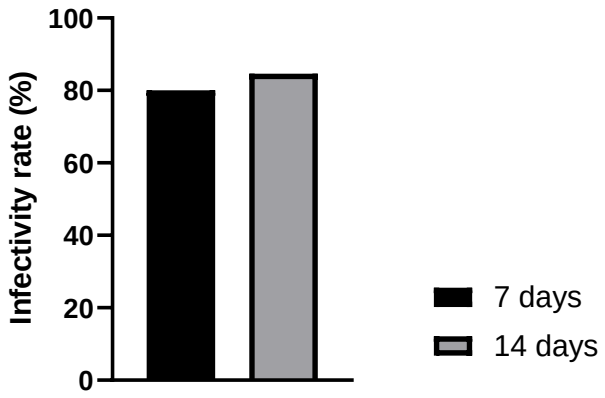


Figure 3.7. Infectivity rate of Rio Grande *Ae. aegypti* after ingesting a blood meal containing 5.5 log $_{10}$ DENV2 (FSB 3381) PFU/ mL at 7 and 14 dpi

Furthermore, the infectivity rates of Rio Grande *Ae. aegypti* mosquitoes for the other strain of DENV- 2 (328298) was 95% (19/20) and 81.25% (26/32) at 7 and 14 dpi. respectively with no significant associations ($p>0.05$) between the infectivity rates and incubation period (**Figure 3.8**).

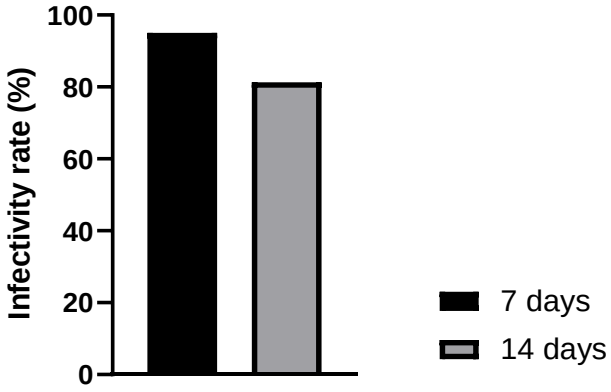


Figure 3.8. Infectivity rate of Rio Grande *Ae. aegypti* after ingesting a blood meal containing 5.5 log $_{10}$ DENV2 (328298) PFU/ mL at 7 and 14 dpi.

Overall, Rio Grande *Ae. aegypti* were susceptible to DENV-2 infection after ingesting a bloodmeal containing 5.5 log $_{10}$ PFU/ mL of either FSB 3381 or 328298 strains (**Figure 3.9)**,

with no differences in the infection rates between the two DENV-2 strains during the 14 days of incubation.

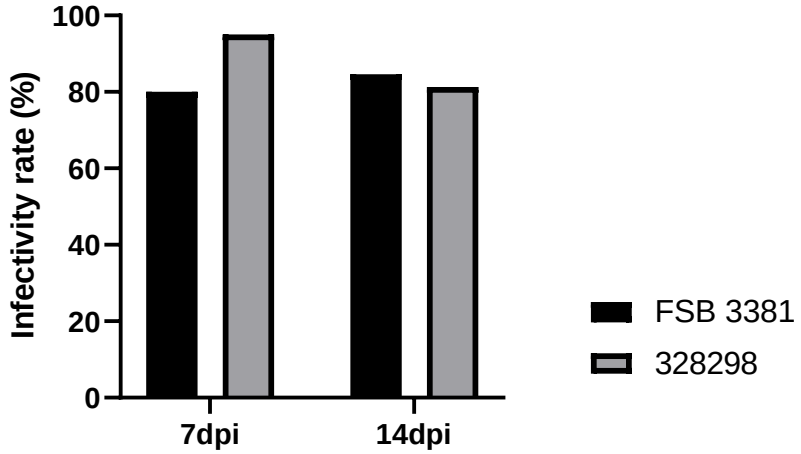


Figure 3.9. Overall infectivity rate of Rio Grande *Ae. aegypti* after ingesting a DENV-2 blood meal containing 5.5 log $_{10}$ PFU/ mL of the DENV-2 (FSB 3381) and DENV2 (328298)

3.2.2.4. DENV-2 replication in *Ae. aegypti* mosquito bodies from the US border

In order to determine dengue viral replication in the infected DENV-2 *Ae. aegypti* mosquitoes from the U.S. border, viral titers in each mosquito were determined by plaque assays through the incubation period.

El Paso *Ae. aegypti* female mosquitoes that ingested 5.5 log $_{10}$ PFU/mL of the DENV-2 (328298) strain had a mean viral titer of 3.03 log $_{10}$ PFU after ingesting the blood/virus mixture (0 dpi). The viral titers in the infected mosquitoes increased to an average of 4.01 log $_{10}$ (ranging from 2.38 to 4.7 log $_{10}$) and 3.92 log $_{10}$ PFU/mL (ranging from 1.6 to 4.6 log $_{10}$ PFU/mL) at 7 and 14 dpi, respectively (**Figure 3.10A**). A significant difference ($p<0.05$) in the mean viral titer at 0 dpi vs 7 dpi, and at 0 dpi vs 14 dpi was observed. No significant difference ($p>0.05$) was observed between the mean viral titers at 7 dpi and 14 dpi. A similar increase in the viral titer in the

infected *Ae. aegypti* mosquitoes during the incubation period was found after ingesting a blood meal of 5 5 log $_{10}$ PFU/ mL of the DENV-2 (FSB 3381) (**Figure 3.10B**). An average viral titer/infected mosquito of 3.52 log $_{10}$ (ranging from 1.18 to 3.92 log $_{10}$ PFU/mL) and 3.96 log (ranging from 1.6 to 4.73 log $_{10}$ PFU/mL) was observed at 7 and 14 dpi, respectively, in comparison to the initial viral titer of 3.19 log $_{10}$ at 0 dpi. The viral titer increased through the incubation period, and a significant difference in the mean viral titer was observed between 7 dpi and 14 dpi ($p<0.05$).

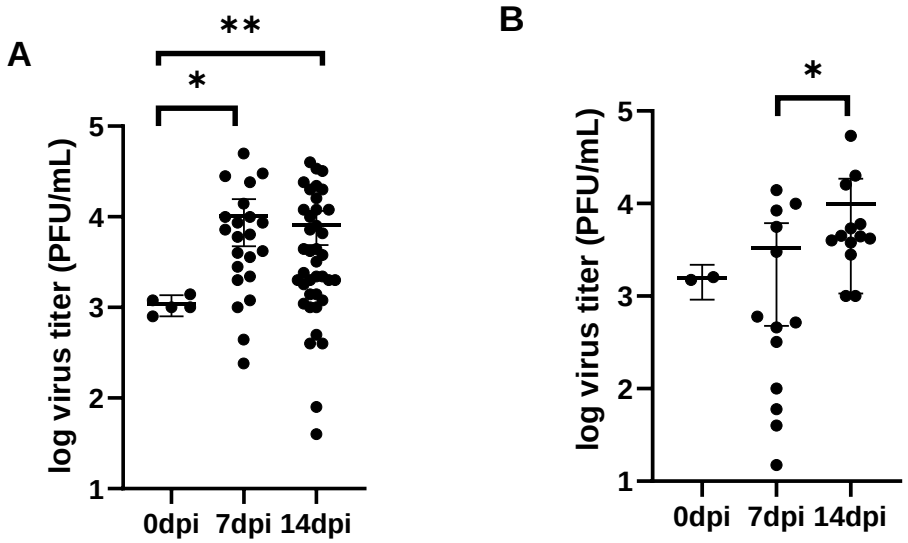


Figure 3.10. DENV-2 replication in *Ae. aegypti* mosquitoes from El Paso at 7 and 14 dpi after ingesting 5.5 log $_{10}$ PFU/ mL of the (**A**) DENV-2 (328298) and (**B**) DENV2 (FSB 3381) strains.

After the Rio Grande *Ae. aegypti* mosquitoes ingested 5.5 log $_{10}$ PFU/mL of DENV-2 (328298) strain, the initial mean DENV-2 titer on day 0 in the engorged mosquitoes was 3.57 log $_{10}$. Viral titer in the infected mosquitoes increased to an average of 4.28 log $_{10}$ PFU/mL (ranging from 2.9 to 4.7 log $_{10}$ PFU/mL) and 4.29 log $_{10}$ PFU/mL (ranging from 2.45 to 4.73 log $_{10}$ PFU/mL) after 7 and 14 dpi, respectively (**Figure 3.11**). Significant differences ($p<0.05$) in the

mean viral titer at 0 dpi vs 7 dpi, and at 0 dpi vs 14dpi were observed. Furthermore, no significant difference was observed in the mean viral titer at 7 dpi vs 14 dpi.

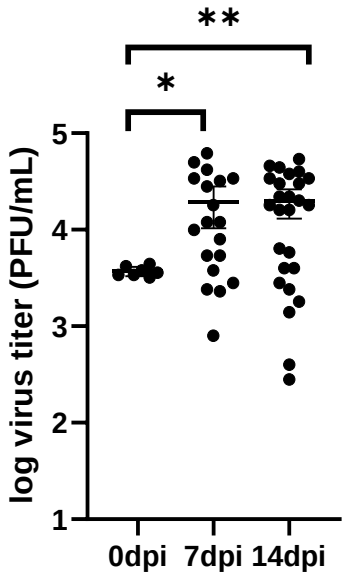


Figure 3.11. DENV-2 replication in *Ae. aegypti* mosquitoes from Rio Grande at 7 and 14 dpi after ingesting 5.5 $\log_{10}$ PFU/ mL of the DENV-2 (328298)

Overall, DENV-2 (FSB 3381) and DENV-2 (328298) strains replicated in El Paso and Rio Grande *Ae. aegypti* mosquitoes through the 14-day incubation period.

3.3. Determine the vector competence of *Ae. aegypti* from the U.S. border region for different strains of DENV-2 based on infection rates, distribution of virus in the body, wings and legs of the mosquitoes and ability to transmit the virus

3.3.1. Material and Methods

3.3.1.1. Standard rearing techniques of *Ae. aegypti*

Ae. aegypti mosquitos' strains were reared at the UTEP Insectary (Biology Building, Room 207) as described in detail previously (Section 3.2.1.1) and maintained at 28ºC, 70% relative humidity and 14h: 10h light: dark cycle (**Table 3.1**).

3.3.1.2. Oral mosquito infections

Third (F3) *Ae. aegypti* generations were tested for DENV-2 oral infection as mentioned previously (Section 3.2.1.4). Briefly, 1 mL of 6 log $_{10}$ PFU/mL of freshly propagated DENV-2 strains was mixed with 2 mL of defibrinated sheep blood (Colorado Serum Company, Denver, CO) and was ingested by female mosquitoes (**Table 3.2**). An aliquot of each infectious blood meal was collected before and after feeding by the mosquitoes to determine the amount of DENV-2 in the virus-blood mixtures that was ingested by the mosquitoes.

After mosquitoes ingested the DENV-2 virus – blood mixture, mosquitoes were cold anesthetized and only the fully engorged females were transferred to a 16 ounces mesh-top cardboard holding cup and fed 10% sucrose in a chamber maintained at 28°C, at 14h: 10h light: dark cycle and 80% humidity for 7 ,14, and 21 days (extrinsic incubation period). In order to determine the amount of DENV-2 ingested by the mosquitoes, two to five engorged females were stored individually at -80°C for plaque assays. At 7, 14, and 21 dpi, batches of individual 20-25 mosquitoes were tested for viral infection.

3.3.1.3. Mosquito processing

Each individual mosquito was processed as follows: abdomen and thorax were examined to estimate infection; legs and wings were examined for dissemination, and saliva was examined for transmission. For saliva collection, the proboscis of each mosquito was inserted into a capillary tube containing 10 μL of mineral oil for salivation for up to 45 min. After salivation, bodies and legs/wing were individually processed in a separate tube as mentioned in the mosquito infectivity section. Briefly, bodies and leg/wings were homogenized in 500 μL of mosquito grinding media (EMEM 2% FBS maintenance medium supplemented with 1% amphotericin B, and 2 % Penicillin/Streptomycin) using a TissueLyzer II (Qiagen, Valencia, CA) for 4 minutes at 30 cycles/second containing a 3 mm tungsten bead per mosquito. Homogenates were clarified by centrifugation at 10,000 g for 10 min at 4ºC and the supernatants were tested to determine viral infectivity in C6/36 cells and titer by plaque assays in BHK 21 cells. Saliva samples were expelled into 100 μL of mosquito grinding media and stored at -80°C until tested for virus. Saliva samples were clarified at 12,000 g for 8 min at 4°C and tested for virus. Only saliva samples from mosquitoes with disseminated infection were tested for virus to determine transmission rates. Furthermore, viral titers of mosquito bodies and legs were determined by plaque assays in BHK-21 cells and expressed as PFU/mL.

3.3.1.4. Viral detection and quantification

Mosquito tissues, including bodies and legs were tested by plaque assays, and IFAT as stated previously (Section 3.2.1.6). In the case of saliva samples, 100μL was inoculated onto a monolayer of C6/36 cells grown in a 96- well plate and observed once daily for CPE daily. After 10 days of incubation, a blind passage was performed into C6/36 cells and cells were maintained

for another 7-10 days at 28°C and 5% CO_2. Later, 20 µL of C6/36 cells were scrapped from the 96-well plate and fixed into ring spotted slides for testing by IFAT to DENV (Section 3.2.1.6.2).

The sensitivity of the IFAT to detect DENV-2 antigen in the mineral oil sample was tested by making serial log dilutions of each DENV-2 strain, then inoculating each dilution into C6/36 cells and performing IFAT to DENV-2. Briefly, DENV-2 strain titers ranging from 0.001 to 100 PFU in 1 µL were spiked into a capillary tube containing 10 µL of mineral oil and processed as a saliva sample (Section 3.3.1.3), then tested for virus by IFAT to DENV-2. The limit of detection of DENV-2 contained in mineral oil was 0.1 PFU, 0.15 PFU, and 1 PFU for FSB 3381, 328298, and 16681 strains, respectively.

3.3.1.5. Evaluation of *Ae. aegypti* strains for Insect-Specific Flavivirus

Ae. aegypti strains used in this study were tested for Insect Specific Flavivirus (ISFV) to determine the possibility that the strains were infected with this virus and therefore could interfere with the results (**Table 3.2**). In nature, ISFV are common among several species of mosquitoes, including *Ae. aegypti* (Blitvich & Firth, 2015). Briefly, F3 generations of *Ae. aegypti*, two to four days old adult mosquitoes (male and female) were pooled in 3 groups of 20 mosquitoes per sex. RNA was extracted from the mosquito suspensions and tested for detection of ISFV RNA by using generic Flavivirus or specific ISFV primers (Contreras-Gutierrez et al., 2017; Kuno et al., 1998). RT-PCR was validated by using viral RNA from ISFVs such as Ae. Flavivirus, Long Pine virus, Culex Flaviviruses among others. In addition, viral isolation in C6/36 cells and IFAT was performed using mouse ascitic fluid to Flaviviruses (SLEV, WNV, ZIKV, DENV-2 and 4G2), Alphaviruses (Chikungunya virus, Western equine encephalitis virus), and Bunyaviruses (La Crosse virus).

3.3.1.6. Statistical Analysis

The infectivity rate was calculated as the proportion of the total number of mosquito bodies tested of the number of virus positive bodies. Disseminated infection rates were calculated as the percent of mosquitoes with both infected bodies and infected legs. Transmission rate was defined as the percent of disseminated infected mosquitoes with virus positive saliva. GraphPad Prism software 8.0 was used for statistical analysis. A Fisher exact test was used to determine the association between infection, disseminated infection rates, and the viral strain and/or the incubation period. Analysis of variance (ANOVA) was used to determine significant differences between viral titers in mosquito bodies, legs, and saliva.

3.3.2. Results

3.3.2.1. El Paso *Ae. aegypti* mosquito's infectivity rate varied according to the DENV-2 strain

El Paso *Ae. aegypti* mosquitoes ingested 6 $\log_{10}$ PFU/mL of DENV-2 strains in different experiments, including DENV-2 (328298), DENV-2 (FSB 3381), and DENV-2 (16681) corresponding to the American, Asian/American, and Asian DENV-2 genotypes, respectively, in order to determine any differences in DENV-2 strains infectivity rates in this mosquito. *Ae. aegypti* mosquitos' bodies (abdomen and thorax) were tested to estimate viral infection and the results revealed differences in the infectivity rates for each DENV-2 strain at different incubation periods (**Figure 3.12**). Infectivity rates ranged from 80% (8/10) to 100% (11/11) and 66.67% (20/30) to 93.3% (28/30) after *Ae. aegypti* ingested DENV-2 (FSB 3381) and DENV-2 (328298) strains, respectively through the incubation period (7 to 21 days). However, low infectivity rates (4.5% [1/22] to 10% [2/20]) were observed in *Ae. aegypti* mosquitoes after ingesting DENV-2 (16681) strain through the 21 days incubation period. There were no significant associations ($p>0.05$) between the different DENV-2 strains infectivity rates and the days post-infection in the El Paso *Ae. aegypti* mosquitoes after ingesting any of the DENV-2 strains, with exception of the infectivity rate at 7 and 14 dpi after ingesting DENV-2 (328298).

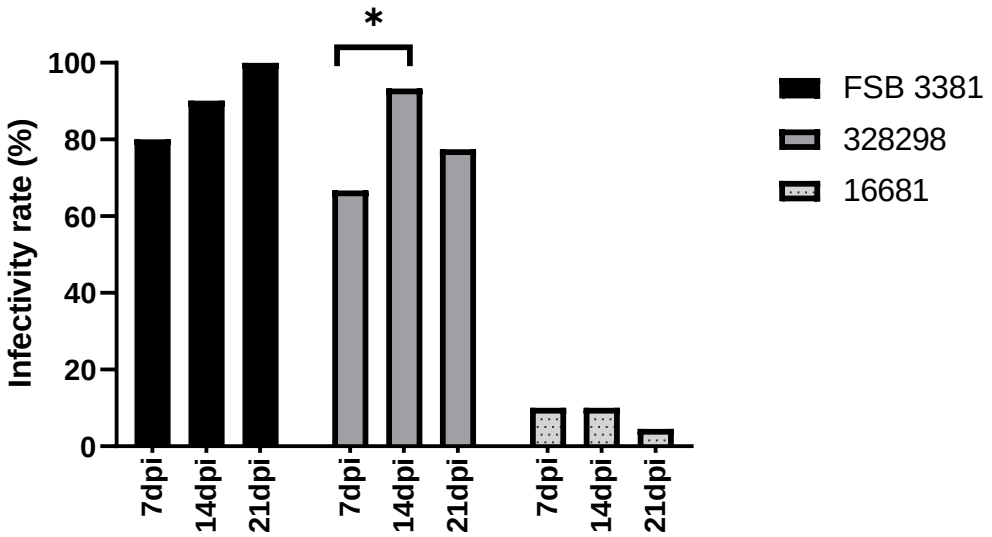


Figure 3.12. Infectivity rate of El Paso *Ae. aegypti* after ingesting DENV-2 in infectious blood meal containing (FSB 3381, 328298 or 16681 strains) of 6 $\log_{10}$ PFU/ mL at 7, 14, and 21 dpi

The infectivity rates of El Paso *Ae. aegypti* after ingesting DENV-2 (16681) was significantly different ($p<0.05$) from the rates of the *Ae. aegypti* mosquitoes that ingested DENV-2 (FSB 3381) and DENV-2 (328298) at 7, 14, and 21 dpi (**Figure 3.13**). No significant differences ($p>0.05$) were noted in the infectivity rates between orally infected *Ae. aegypti* for DENV-2 (FSB 3381) and DENV-2 (328298) through the incubation period of 21 days.

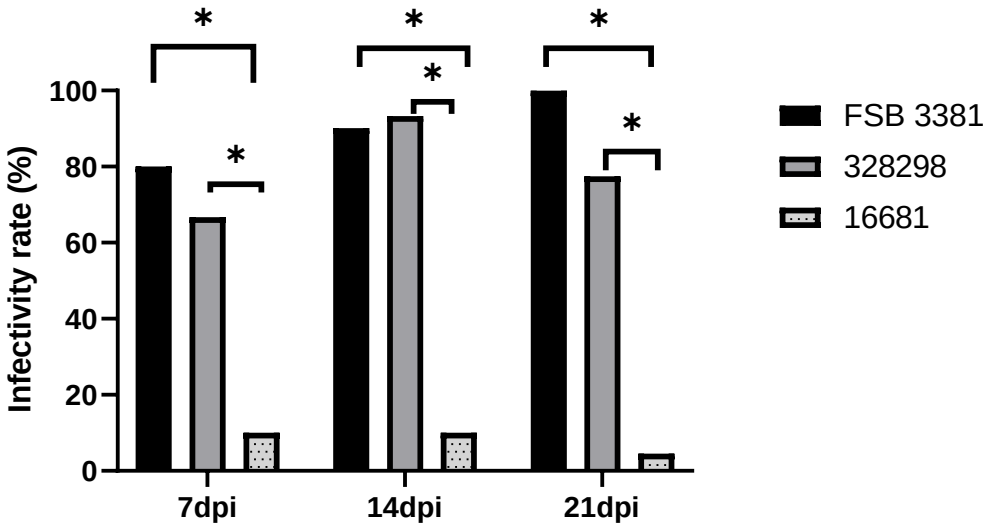


Figure 3.13. Comparison of the infectivity rate in El Paso *Ae. aegypti* after ingesting DENV-2 strains (FSB 3381, 328298,16681) of 6 $\log_{10}$ PFU/ mL at 7, 14, and 21 dpi.

3.3.2.2. DENV-2 (FSB 3381) and DENV-2 (328298) strains caused a disseminated infection in El Paso *Ae. aegypti* mosquitoes

El Paso *Ae. aegypti* mosquito legs and wings of mosquito bodies that were positive for DENV-2 (previously determined by the infectivity rate) were tested by plaque assay and viral isolation to determine the viral disseminated infection rates. At 7 dpi, disseminated infection rates were 40% (8/20) and 62.5% (5/8) for DENV-2 (328298) and DENV-2 (FSB 3381), respectively (**Figure 3.14**). At 14 dpi, DENV-2 (328298) disseminated infection rates increased significantly ($p<0.05$) to 85.71% (24/28) in comparison to 7 dpi, while DENV-2 (FSB 3381) disseminated infection rates reached 100% (10/10) and almost reached statistical significance ($p=0.069$) in comparison to the 62.5% (5/8) at 7dpi. Disseminated infection rates at 21 dpi were 87.5% (21/24) and 100% (11/11) for DENV-2 (328298) and DENV-2 (FSB 3381), respectively, and these rates did not differ significantly ($p>0.05$) in comparison to disseminated infection rates at 14 dpi for DENV-2 (328298) or DENV-2 (FSB 3381) infected mosquitoes. Also, disseminated

infection rates comparison between 7 dpi and 21 dpi showed an increase through time in the DENV-2 (FSB 3381) ($p=0.058$) and DENV-2 (328298) ($p<0.05$) infected mosquitoes.

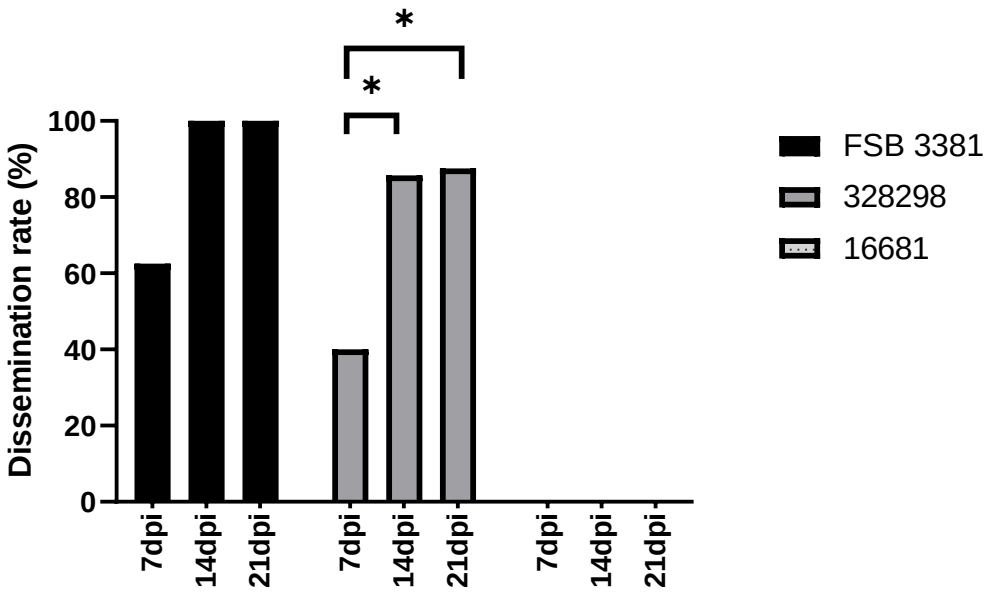


Figure 3.14. Disseminated infection rates for El Paso *Ae. aegypti* after ingesting a DENV-2 infectious blood meal containing (FSB 3381, 328298,16681) of 6 $\log_{10}$ PFU/ mL at 7, 14, and 21 dpi.

On the other hand, none of the few *Ae. aegypti* (n=5) mosquitoes infected with DENV-2 (16681) had evidence of virus dissemination to the legs and wings through the 21 days incubation period, and therefore, the disseminated infection rate was 0% (0/5). Overall, DENV-2 disseminated infection rates in infected El Paso *Ae. aegypti* mosquitoes increased through time and reached a maximum rate at 14 dpi after ingesting DENV-2 (FSB 3381) and DENV-2 (328298) strains. Furthermore, no significant differences ($p>0.05$) were noted between the disseminated infection rates of the El Paso *Ae. aegypti* mosquitoes after ingesting DENV-2 (FBS 3381) and DENV-2 (328298) strains through 21 dpi (**Figure 3.15**). As stated before, a null disseminated infection rate was found for DENV-2 (16681) strain and statistical analysis was not

performed with the other DENV-2 strains, due to the low number of mosquitoes bodies infected with DENV-2 (16681) strain (2 bodies at 7 dpi, 2 bodies at 14 dpi, and 1 body at 21 dpi).

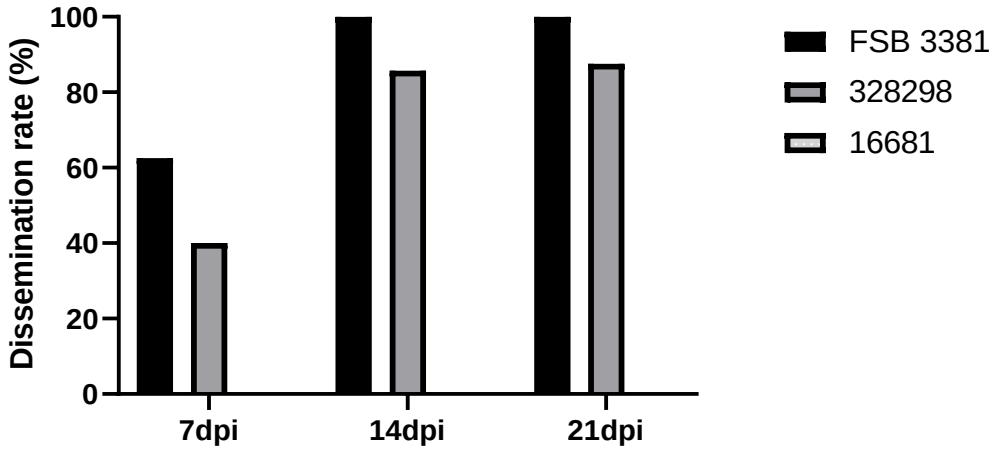


Figure 3.15. Comparison of the disseminated infection rate in El Paso *Ae. aegypti* after ingesting DENV-2 strains (FSB 3381, 328298, 16681) of 6 $\log_{10}$ PFU/ mL at 7, 14, and 21 dpi

3.3.2.3. Attempts to detect virus in the saliva from DENV-2 disseminated El Paso *Ae. aegypti* mosquitoes

Saliva samples collected from mosquitoes that had disseminated infection with DENV was tested for viral infectivity in C6/36 cells. Overall, no infective virus was detected in any of the 26 *Ae. aegypti* mosquito saliva samples that had disseminated infections with DENV-2 (FSB 3381) (**Table 3.3.**) In the case of the DENV-2 (328298) disseminated infected mosquitoes, DENV-2 was detected in only one saliva sample out of 53 samples (1.89%). No saliva samples were tested from *Ae aegypti* mosquitoes after ingesting DENV-2 (16681) due to the absence of disseminated infection with DENV-2 (16681).

Table 3.3. DENV-2 transmission rate in El Paso *Ae. aegypti* after ingesting DENV-2 strains (FSB 3381, 328298) of 6 $\log_{10}$ PFU/ mL at 7, 14, and 21 dpi

	FSB 3381	328298
	% (*No Pos/ &No Dis)	
7dpi	0 (0/5)	0 (0/8)
14dpi	0 (0/10)	4.17 (1/24)
21dpi	0 (0/11)	0 (0/21)
Total	0 (0/26)	1.89 (1/53)

*No Pos= DENV-2 positive saliva sample

&No Dis= Number of DENV-2 disseminated

3.3.2.4. Dengue virus replication within *Ae. aegypti* mosquitos' bodies and legs from the El Paso varied according to the viral strain

The dengue viral titer in the *Ae. aegypti* bodies and legs/wings tissues harvested through the incubation period (7 to 21 days) were determined by plaque assays in BHK-21 cells. Briefly, the mean viral titer in the El Paso mosquito bodies infected with DENV-2 (FSB 3381) was 3.537 $\log_{10}$, 3.329 $\log_{10}$, and 4.206 $\log_{10}$ PFU/mL at 7, 14, and 21 dpi, respectively (**Figure 3.16A**). No significant difference was observed in the mean viral titers up to 14 dpi; however, a significant increase was observed from 3.329 to 4.206 $\log_{10}$ PFU/mL at 14 and 21 dpi, respectively ($p<0.05$). In case of the mosquito's bodies infected with DENV-2 (328298), the mean viral titers were 4.059 $\log_{10}$, 3.412 $\log_{10}$, and 3.819 $\log_{10}$ PFU/mL at 7, 14, and 21 dpi, respectively. Significant differences were found between 7 dpi (4.059 $\log_{10}$ PFU/mL) and 14 dpi (3.412 $\log_{10}$ PFU/mL) mean viral titers ($p<0.05$) (**Figure 3.16B**). In contrast, *Ae aegypti* mosquitoes infected with DENV-2 (16681) had low viral titers by plaque assays with mean body titer of 2.688 $\log_{10}$, 2.159 $\log_{10}$, and 2.255 $\log_{10}$ PFU/mL at 7, 14, and 21dpi, respectively (**Figure 3.16C**).

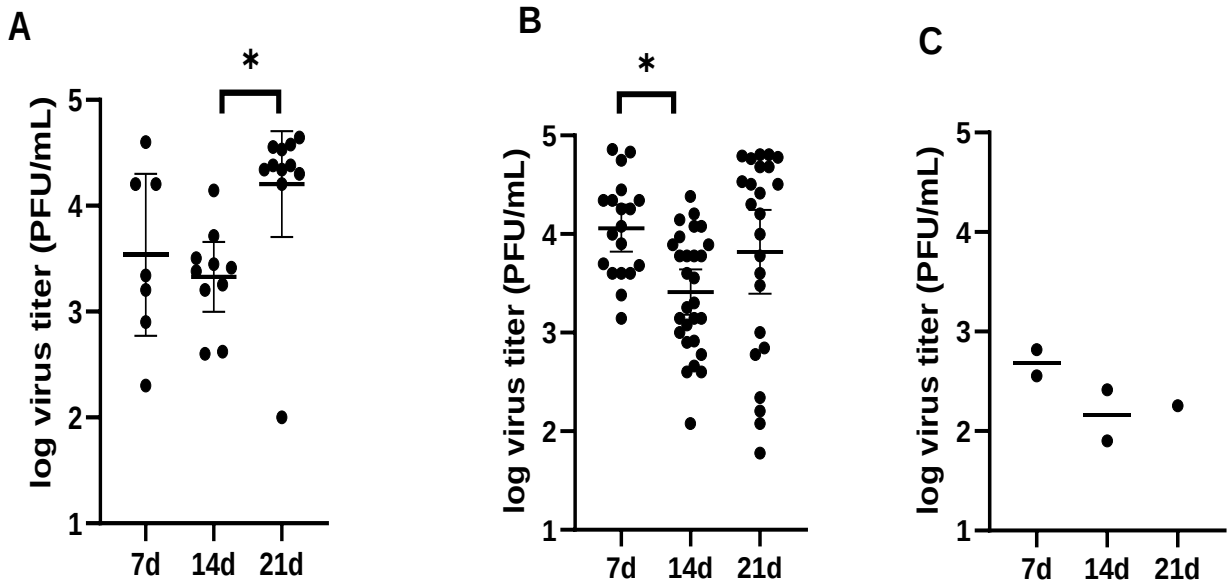


Figure 3.16. DENV-2 replication in El Paso *Ae. aegypti* mosquito bodies at 7, 14, and 21 days after ingesting 6 log $_{10}$ PFU/ mL of the (**A**) DENV-2 (FSB 3381), (**B**) 328298, and (**C**) 16681 strains.

The mean viral titers in *Ae. aegypti* legs of confirmed DENV infection in their bodies, varied through the incubation period. Briefly, legs from mosquitoes infected with DENV-2 (FSB 3381) strain had a mean viral titer of 1.805 log $_{10}$, 1.805 log $_{10}$, and 2 log $_{10}$ PFU/mL at 7, 14, and 21 dpi, respectively (**Figure 3.17A**), and there was no significant difference ($p>0.05$) between mean viral titer in the legs and the incubation period. Furthermore, legs of mosquitoes that ingested DENV-2 (328298) had a mean titer of 1,925 log $_{10}$, 2.096 log $_{10}$, and 2.384 log $_{10}$ PFU/mL at 7,14, and 21 dpi, respectively (**Figure 3.17B**). Even though an increase in mean leg viral titer was observed, no significant difference was noted. Furthermore, no plaque forming units were detected in any of the *Ae. aegypti* legs with infectious DENV-2 (16681) bodies.

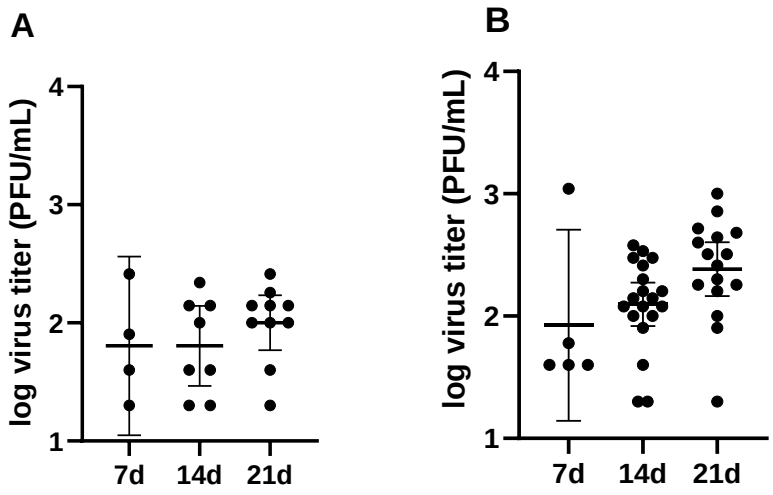


Figure 3.17. DENV-2 replication in El Paso *Ae. aegypti* mosquito legs at 7, 14, and 21 days after ingesting 6 log $_{10}$ PFU/ mL of (**A**) DENV-2 (FSB 3381) and (**B**) DENV-2 (328298).

Overall, the replication of DENV-2 (FSB 3381) and DENV-2 (328715) strains in El Paso *Ae. aegypti* mosquito bodies and legs slightly increase through time with some significant changes. Furthermore, the DENV-2 (16681) strain was unable to replicate efficiently in El Paso *Ae. aegypti* mosquito bodies and therefore, prevented the replication of the virus in the mosquito legs tissues.

3.3.2.5. Rio Grande *Ae. aegypti* mosquito's infectivity rate varied according to the DENV-2 strain

Rio Grande *Ae. aegypti* mosquitoes were allowed to ingest 6 log $_{10}$ PFU/mL of DENV-2 strains (328298, FSB 3381, and 16681). *Ae. aegypti* mosquito bodies following ingestion of DENV-2 (FSB 3381) had and infectivity rate of 60.87% (14/23), 91.3% (21/23), and 87.5% (21/24) at 7, 14, and 21 dpi, respectively (**Figure 3.18**). A significant increase ($p<0.05$) in the infectivity rate was observed through the incubation period, including days 7, 14, and 21. In case of the *Ae. aegypti* mosquitoes that ingested DENV-2 (328298), infectivity rates were 79.92%

(20/26), 76% (19/25), and 96.15% (25/26) at 7, 14, and 21 incubation days, respectively, with a significant difference ($p<0.05$) in the infectivity rates at 14 vs 21 incubation days. Finally, *Ae. aegypti* mosquitos exposed to DENV-2 (16681) had lower infectivity rates, in comparison to the other DENV-2 strains, ranging from 9.1% (2/22) to 13.6% (3/22) through the incubation period with no significant differences through the incubation period.

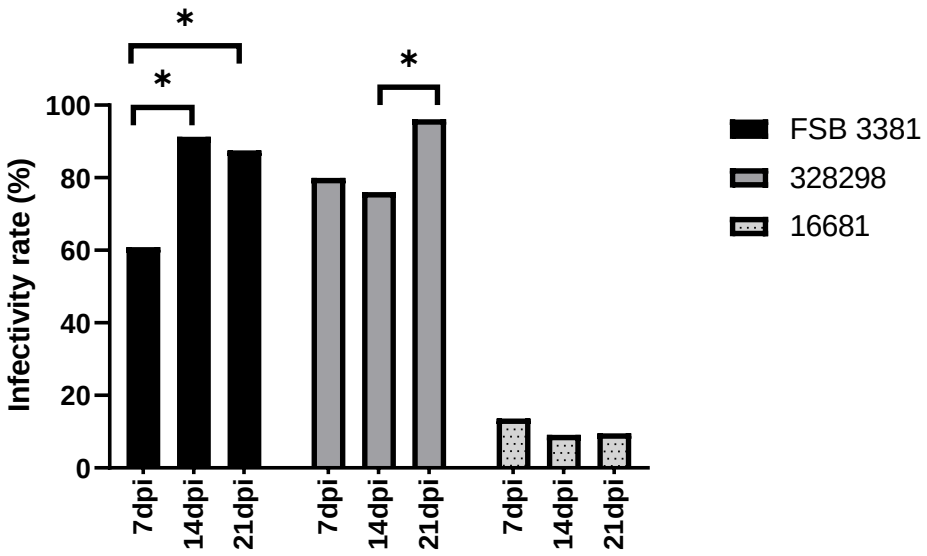


Figure 3.18. Infectivity rate of Rio Grande *Ae. aegypti* after ingesting DENV-2 strains (FSB 3381, 328298,16681) of 6 $\log_{10}$ PFU/ mL at 7, 14, and 21 dpi

A comparison of the infectivity rates between each DENV-2 strains ingested by Rio Grande *Ae. aegypti* showed a significant decrease ($p<0.05$) in the DENV-2 (16681) infectivity rate in comparison to the rates for DENV-2 (FSB3381) and DENV-2 (328298) at 7, 14, and 21 dpi (**Figure 3.19**). Also, no significant difference ($p>0.05$) was observed in the infectivity rate between the DENV-2 (FSB 3381) and DENV-2 (328298) strains.

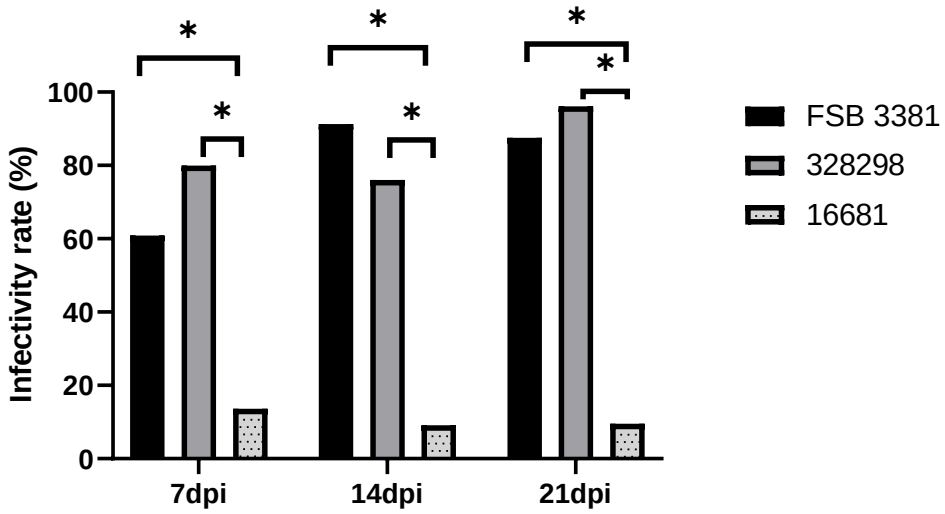


Figure 3.19. Comparison of the infectivity rate in Rio Grande *Ae. aegypti* after ingesting DENV-2 strains (FSB 3381, 328298,16681) of 6 log $_{10}$ PFU/ mL at 7, 14, and 21 dpi

3.3.2.6. DENV-2 disseminating infections in Rio Grande *Ae. aegypti* mosquitoes

Ae. aegypti mosquitoes from Rio Grande infected with the different DENV strains had increased disseminated infection rates through the incubation period (**Figure 3.20**). Disseminated infection rates for *Ae. aegypti* that ingested DENV-2 (FSB 3381) were 60.87% (14/23), 90.48% (19/21), and 85.71% (18/21) at 7, 14, and 21 dpi, respectively. Similarly, disseminated infection rates of *Ae. aegypti* that ingested DENV-2 (328298) strain were 10% (2/20), 73.68% (14/19), and 80% (20/25) at 7, 14, and 21 dpi, respectively. A significant increase ($p<0.05$) in the disseminated infection rate was observed between 7 dpi and 14 dpi, and 7 dpi and 21 dpi. Finally, disseminated infection rates of *Ae. aegypti* that ingested DENV-2 (16681) was 33% (1/3), 100% (2/2), and 50% (1/2) at 7,14, and 21dpi but no statistical analysis was observed due to the limited number of mosquitoes.

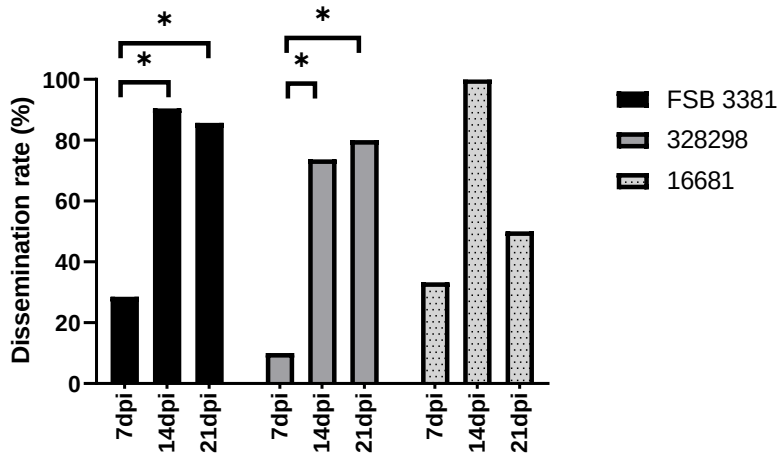


Figure 3.20. Disseminated infection rates of Rio Grande *Ae. aegypti* after ingesting DENV-2 blood meal (FSB 3381, 328298,16681) containing 6 $\log_{10}$ PFU/ mL at 7, 14, and 21 dpi.

Disseminated infection rates of *Ae. aegypti* that ingested DENV-2 (FSB 3381) and DENV-2 (328298) strains were significantly different at 7 dpi only (**Figure 3.21**) but were not significantly different at 14 and 21 dpi. Unlike El Paso *Ae. aegypti* mosquitoes that didn't have disseminated infection rates for DENV-2 (16681), few Rio *Ae. aegypti* (n=4) had infective virus in the legs through 21 dpi as mentioned previously.

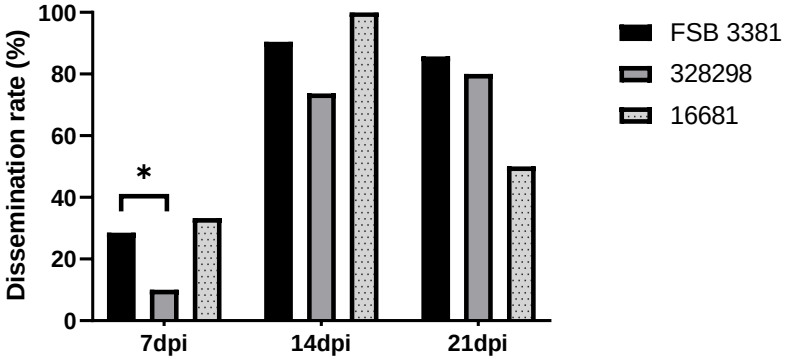


Figure 3.21. Comparison of the disseminated infection rate in Rio Grande *Ae. aegypti* after ingesting DENV-2 strains (FSB 3381, 328298) of 6 $\log_{10}$ PFU/ mL at 7, 14, and 21 dpi

3.3.2.7. Detection of infective virus in saliva from DENV-2 disseminated Rio Grande *Ae. aegypti* mosquitoes

DENV-2 was isolated from the mosquito saliva in *Ae. aegypti* that ingested DENV-2 (FSB 3381) and DENV-2 (328298) strains (**Table 3.4.**). The overall transmission rates for *Ae. aegypti* that ingested DENV-2 (FSB3381) and DENV-2 (328298) strains were 2.44% (1/41) and 2.78% (1/36), respectively. No virus was detected in any of the 4 saliva samples from *Ae. aegypti* mosquitoes with a disseminated DENV-2 (16681) infection.

Table 3.4. DENV-2 transmission rate for Rio Grande *Ae. aegypti* after ingesting DENV-2 strains of 6 $\log_{10}$ PFU/ mL at 7, 14, and 21 dpi.

	FSB 3381	328298	16681
	% (*No Pos/ &No Dis)		
7dpi	0 (0/4)	0 (0/2)	0 (0/1)
14dpi	5.26 (1/19)	0 (0/14)	0 (0/2)
21dpi	0 (0/18)	0 (1/20)	0 (0/1)
Total	2.44 (1/41)	2.78 (1/36)	0 (0/4)

*No Pos= DENV-2 positive saliva sample

&No Dis= Number of DENV-2 disseminated

3.3.2.8. Dengue virus replication within Rio Grande *Ae. aegypti* mosquitos' bodies and legs varied according to the viral strain

Viral replication of each DENV-2 strain within Rio Grande *Ae. aegypti* mosquito bodies was determined by plaque assays. The mean viral titer in Rio *Ae. aegypti* bodies infected with DENV-2 (FSB 3381) was 3.421 $\log_{10}$, 3.617 $\log_{10}$, and 3.784 $\log_{10}$ PFU/mL at 7, 14, and 21 dpi respectively (**Figure 3.22A**). Even though an increase in the mean mosquito body viral titer was detected over time, no significant differences in the mean titer were associated with the time of incubation. *Ae. aegypti* mosquito bodies infected with DENV-2 (328298) had a mean viral titer

of 3.559 log $_{10}$, 3.648 log $_{10}$, and 4,181 log $_{10}$ PFU/mL at 7, 14, and 21 dpi (**Figure 3.22B**). Significant differences ($p<0.05$) were found between mean viral titer at 7 dpi (3.559 log $_{10}$) vs 21 dpi (4,181 log $_{10}$), and 14 dpi (3.648 log $_{10}$ PFU/mL) and 21 dpi (4,181 log $_{10}$ PFU/mL). Finally, only a few *Ae. aegypti* mosquitoes exposed to DENV-2 (16681) had virus in their bodies (**Figure 3.22C**). Based on the limited number (n=7) during the incubation period, an estimated mean viral titer of 3.678 log $_{10}$, 5.007 log $_{10}$, and 3.371 log $_{10}$ PFU/mL was found at 7, 14, and 21 dpi.

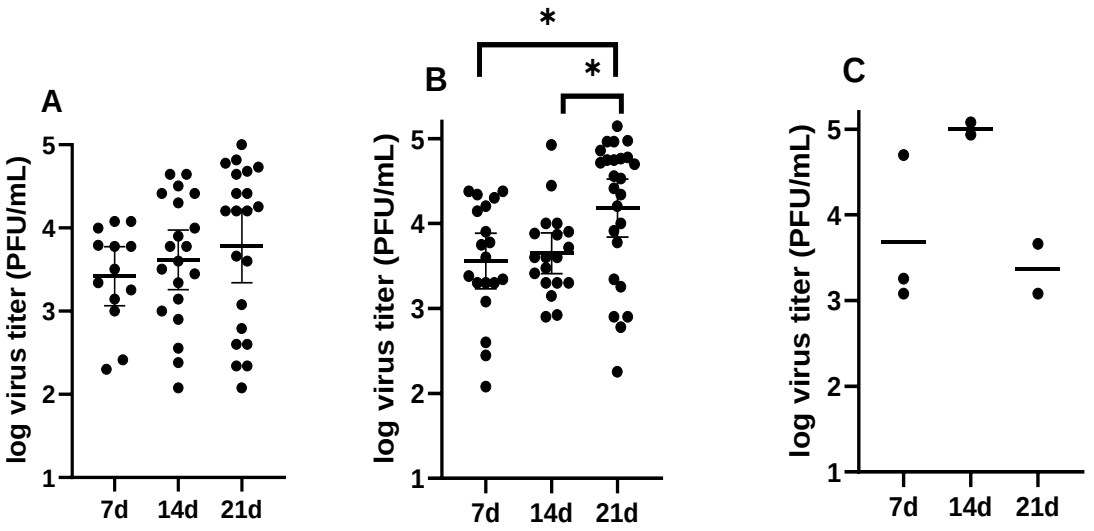


Figure 3.22. DENV-2 replication of *Ae. aegypti* mosquito bodies from Rio Grande at 7, 14, and 21 days after ingesting 6 log $_{10}$ PFU/ mL of the DENV-2 (FSB 3381), 328715, and 16681 strains.

DENV-2 titer in *Ae. aegypti* legs varied through the incubation period. Briefly, legs from mosquitoes that ingested DENV-2 (FSB 3381) strain had a mean viral titer of 2.358 log $_{10}$, 2.414 log $_{10}$, and 2.141 log $_{10}$ PFU/mL at 7, 14, and 21 dpi, respectively (**Figure 3.23A**). No significant differences ($p>0.05$) were found between the mean viral titer through the incubation period. In addition, legs from mosquitoes that ingested DENV-2 (328298) had a titer of 2 log $_{10}$, 2.736 log $_{10}$, and 3.144 log $_{10}$ PFU/mL at 7, 14, and 21 dpi, respectively. A significant increase in the mean

viral titer ($p<0.05$) was noted between 14 dpi (2.736 log $_{10}$ PFU/mL) and 21dpi (3.144 log $_{10}$ PFU/mL) (**Figure 3.23B)**. Finally, a viral titer ranging from 2.477 to 4.041 log $_{10}$ PFU/mL was detected in the *Ae. aegypti* legs infected with DENV-2 (16681) (**Figure 23C**).

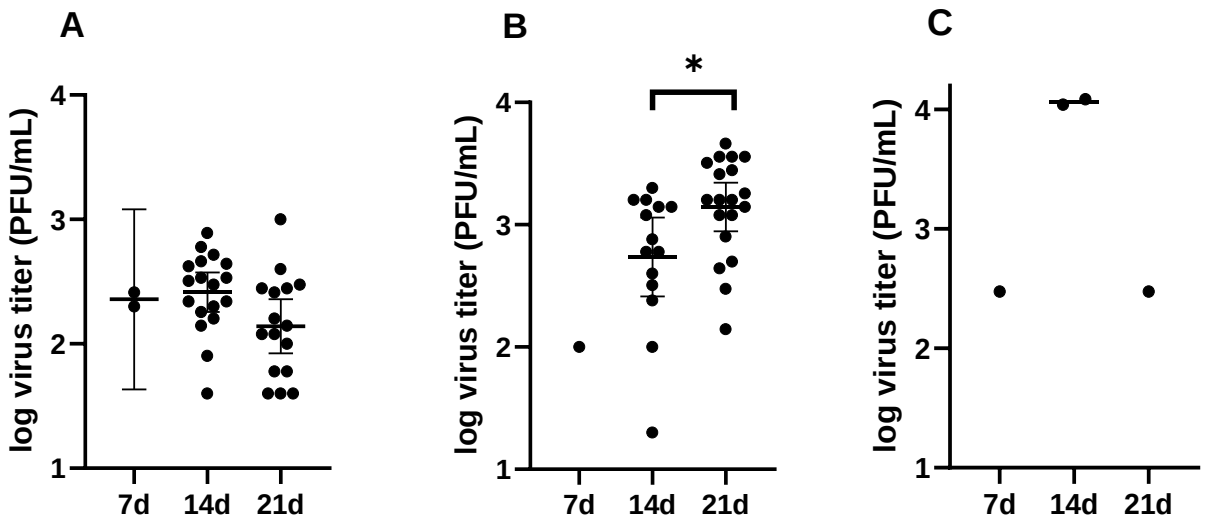


Figure 3.23. DENV-2 replication in *Ae. aegypti* mosquito legs from Rio Grande at 7, 14, and 21 dpi after ingesting 6 log $_{10}$ PFU/ mL of the (**A**) DENV-2 (FSB 3381), (**B**) 328298, and (**C**)16681.

Finally, two of six *Ae. aegypti* pool suspensions from Rio Grande were positive by RT-PCR for ISFVs, while El Paso *Ae. aegypti* mosquitoes were negative for ISFVs. Partial sequencing of the Flavivirus NS5 region identified a sequence of Cell Fusing Agent virus (CFAV) in those 2 pools. Furthermore, after serial passages in C6/36 cells, the CFAV was isolated from one sample.

3.4. Determine the effect of a low DENV-2 dose on the vector competence of *Ae. aegypti* from the U.S. border

3.4.1. Material and Methods

3.4.1.1. Standard rearing techniques of *Ae. aegypti*

Ae. aegypti mosquito strains were reared in the UTEP Insectary (Biology Building, Room 207) as described in detail in the above section (Section 3.2.1.1) (**Table 3.1.**).

3.4.1.2. Oral mosquito infections

Third (F3) *Ae. aegypti* generations were infected with DENV-2 as mentioned in the previous section (Section 3.2.1.4). Briefly, 1mL of 4 $\log_{10}$ PFU/mL (originally diluted from a freshly propagated DENV-2 stock of 6 $\log_{10}$ PFU/mL) was mixed with 2 mL of defibrinated sheep blood (Colorado Serum Company, Denver, CO) and ingested by female *Ae. aegypti* mosquitoes from El Paso and Rio Grande.

3.4.1.3. Mosquito processing

Each mosquito was tested as follows: abdomen and thorax were tested to determine possible infection in mosquito legs and wings for disseminated infections; and saliva for virus transmission (Section 3.3.1.3).

3.4.1.4. Viral detection and quantification

Mosquito bodies, legs, and saliva were tested by plaque assays for virus in C6/36 cells and identified by IFAT as described previously (Section 3.2.1.6).

3.4.1.5. Statistical Analysis

Statistical analysis was performed as described above (Section 3.3.1.6). Furthermore, the oral dose of a DENV-2 strain required to infect 50% mosquitoes MID50) was calculated where possible, utilizing the method of Reed and Muench (Reed & Muench, 1938).

3.4.2. Results

3.4.2.1 Vector competence of El Paso *Ae. aegypti* mosquitoes after ingesting 4 log $_{10}$ PFU/mL DENV-2

Overall, the infectivity and disseminated infection rates of El Paso mosquitoes that ingested 4 log $_{10}$ PFU/mL of virus are presented in **Figure 3.24**. Infectivity rates of 85% (17/20), 75% (18/24), and 79.17% (19/24) were obtained at 7, 14, and 21dpi, respectively after mosquitoes ingested 4 log $_{10}$ PFU/mL of the DENV-2 (FSB 3381) (**Figure 3.24A**). In case of the mosquitoes that ingested DENV-2 (328298), the infectivity rates were 45% (9/20), 30% (6/20), and 50% (12/24) at 7, 14, and 21 dpi, respectively.

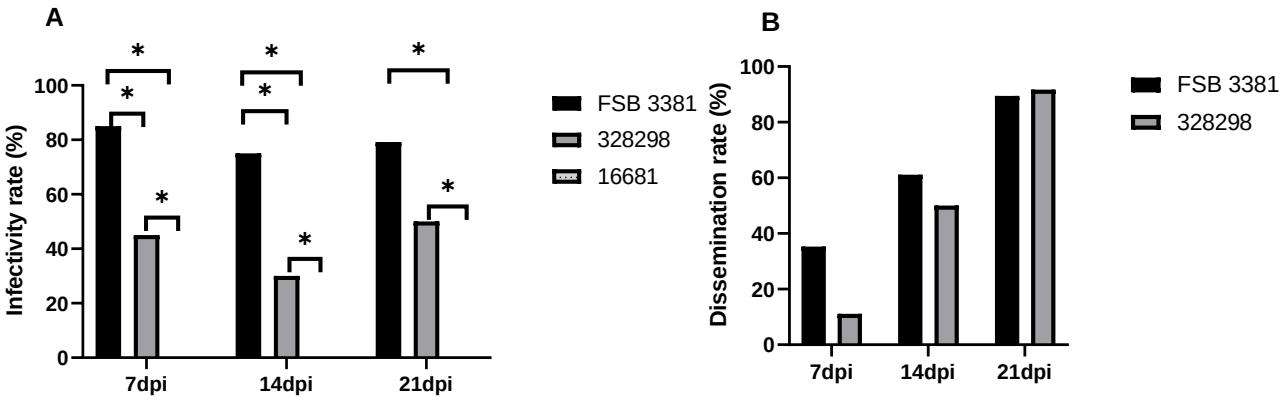


Figure 3.24. Comparison of the (**A**) infectivity and (**B**) disseminated infection rates in El Paso *Ae. aegypti* after ingesting DENV-2 strains (FSB 3381, 328298, 16681) of 4 log $_{10}$ PFU/ mL at 7, 14, and 21 dpi.

Mosquitoes were not infected after ingesting 4 log $_{10}$ PFU/mL of DENV-2 (16681) (infectivity rate of 0% [0/20]). Overall, the infectivity rate was not affected by the incubation period, but there was a significant difference ($p<0.05$) in the infectivity rate between each DENV-2 strain (FSB 3381 vs 328298, FSB 3381 vs 16681, 32898 vs 16681) during the

incubation period. The highest infectivity rate among the 3 DENV-2 strains was for the *Ae. aegypti* mosquitoes infected with DENV-2 (FSB 3381) strain.

In regard to disseminated infection, mosquitoes that ingested DENV-2 (FBS 3381) and DENV-2 (328298) strains had a significant increase in the disseminated infection rates through the incubation period ($p<0.05$) (**Figure 3.24B**). E.g., DENV-2 (FSB 3381) disseminated infection rates were 35.29% (6/17) and 89.47% (17/19) at 7 and 21 dpi respectively, while DENV-2 (328298) disseminated infection rates were 11.11 (1/9) and 91.67 (11/12) at 7 and 21 dpi respectively. Furthermore, no significant difference in the disseminated infection rates were noted between DENV-2 (FSB 3381) and DENV-2 (328298). Legs were not tested for mosquitoes that ingested the DENV-2 (16681) strain because infection was not observed in the mosquito bodies.

Virus was not detected in any of the mosquito saliva samples that were obtained from *Ae. aegypti* that ingested 4 $\log_{10}$ PFU/mL of the DENV-2 (FSB 3381) and DENV-2 (328298) strains (**Table 3.5.**). No saliva samples were tested from mosquitoes that ingested DENV-2 (16681) due to the null infection observed.

Table 3.5. DENV-2 transmission rate by El Paso *Ae. aegypti* after ingesting DENV-2 strains (FSB 3381, 328298) of 4 $\log_{10}$ PFU/ mL at 7, 14, and 21 dpi.

	FSB 3381	328298
	% (*No Pos/ &No Dis)	
7dpi	0 (0/6)	0 (0/1)
14dpi	0 (0/11)	0 (0/3)
21dpi	0 (0/17)	0 (0/11)
Total	0(0/34)	0 (0/15)

*No Pos= DENV-2 positive saliva sample

&No Dis= Number of DENV-2 disseminated

3.4.2.2. Vector competence of Rio Grande *Ae. aegyti* mosquitoes after ingesting 4 log $_{10}$ PFU/mL DENV-2

Infectivity rates for Rio Grande *Ae. aegypti* mosquitoes after ingesting 4 log $_{10}$ PFU/mL of DENV-2 (FSB 3381) were 25% (6/24), 25% (6/24), and 47.8% (11/23) at 7,14, and 21dpi, respectively, while the infectivity rates after exposure to DENV-2 (328298) were 15.38% (4/26), 30.43% (7/23) and 8.6% (2/23) at 7,14 and 21 dpi, respectively (**Figure 3.25A**). No infection (0%, 0/72) was observed in mosquitoes that ingested 4 log $_{10}$ PFU/mL DENV2 (16681) during the 21 days incubation period No significant differences were observed in the infectivity rates during the incubation period. However, significant differences ($p<0.05$) in the infectivity rates were associated mainly between DENV-2 (FSB 3381) and DENV-2 (16681) strains during the incubation period (7 to 21dpi). Other significant differences in the infectivity rates occurred between DENV-2 (328298) and DENV-2 (16681) strains at 14 dpi, and between DENV-2 (FSB 3381) and DENV-2 (328298) strains at 21 dpi.

Disseminated infection rates for Rio Grande mosquitoes that ingested DENV-2 (FSB 3381) and DENV-2 (328298) increased through the incubation period (**Figure 3.25B**). Disseminated infection rates after ingesting DENV-2 (FSB 3381) were 16.67% (1/6), 50% (3/6), and 81.81% (9/11) at 7,14, and 21 dpi, respectively with the disseminated infection rate being significantly different at 7 dpi vs 21 dpi. In case of mosquitoes that ingested DENV-2 (328298), the disseminated infection rates were 50% (2/4), 71.43% (5/7), and 100% (2/2) at 7,14, and 21 dpi, respectively. Also, because mosquitoes that ingested DENV-2 (16681) did not become infected, mosquito legs were not tested for a disseminated infection. Finally, there were no significant differences in the disseminated infection rates between DENV-2 (FSB 3381) and DENV-2 (328298) strains at 7, 14, and 21dpi.

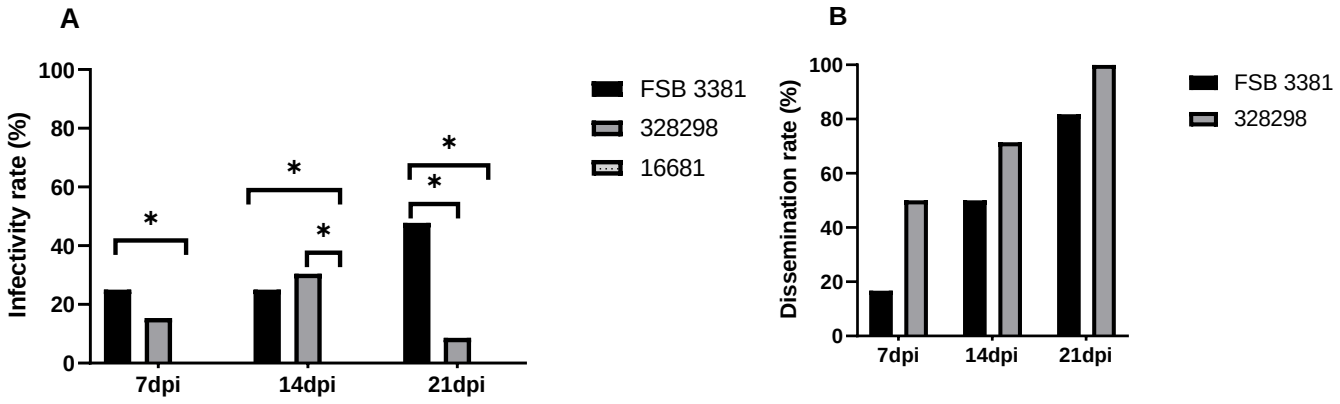


Figure 3.25. Comparison of the (**A**) infectivity and (**B**) disseminated infection rates for El Paso *Ae. aegypti* after ingesting DENV-2 strains (FSB 3381, 328298) of 4 $\log_{10}$ PFU/ mL at 7, 14, and 21 dpi.

Virus was detected in the saliva of only 1 of 13 (7.69%) mosquitoes that had a DENV-2 (FSB 3381) disseminated infection (**Table 3.6.**). No virus was detected in the saliva of the 9 mosquitoes with a disseminated DENV-2 (328298) infection.

Table 3.6. DENV-2 transmission rate in El Paso *Ae. aegypti* after ingesting DENV-2 strains (FSB 3381, 328298) of 4 $\log_{10}$ PFU/ mL at 7, 14, and 21 dpi

	FSB 3381	328298
	% (*No Pos/ [&]No Dis)	
7dpi	0 (0/1)	0 (0/2)
14dpi	0 (0/3)	0 (0/5)
21dpi	11.1 (1/9)	0 (0/2)
Total	7.69(1/13)	0 (0/9)

*No Pos= DENV-2 positive saliva sample

[&]No Dis= Number of DENV-2 disseminated

3.4.2.3. DENV-2 replication in El Paso and Rio Grande *Ae. aegypti* mosquitoes after ingesting 4 log $_{10}$ PFU/mL of DENV-2

El Paso *Ae. aegypti* mosquitoes that ingested 4 log $_{10}$ of DENV-2 (FSB 3381) strain had a log $_{10}$ mean body viral titer of 3.249 log $_{10}$, 2.981 log $_{10}$, 3.567 log $_{10}$ PFU/mL at 7, 14, and 21 dpi, respectively, with no significant differences in the mean titer through the period of incubation (**Figure 3.26A**). El Paso mosquitoes that ingested DENV-2 (328298) strain had a mean body log titer of 2.761 log $_{10}$, 2.684 log $_{10}$, 3.722 log $_{10}$ PFU/mL at 7,14, and 21 dpi, respectively (**Figure 3.26B**). A significant difference ($p<0.05$) in the mean viral titer ($p<0.05$) was observed between 7 dpi and 21 dpi, and between 14dpi and 21dpi. The mean leg viral titer in El Paso mosquito legs after ingesting o DENV-2 (FSB 3381) was 1.835 log $_{10}$, 1.856log $_{10}$, 1.951 log $_{10}$ PFU/mL at 7, 14, and 21 dpi, respectively (**Figure 3.26C**), with no significant differences in the mean leg viral titer through the incubation period. The mean viral titer in mosquito's legs for mosquitoes that ingested DENV-2 (328298) had a slight increase through time and the titer was 1.301 log $_{10}$, 2.094 log $_{10}$, and 2.220 log $_{10}$ PFU/mL at 7, 14, and 21dpi, respectively (**Figure 3.26D**). However, no significant difference was observed in the mean titer through time.

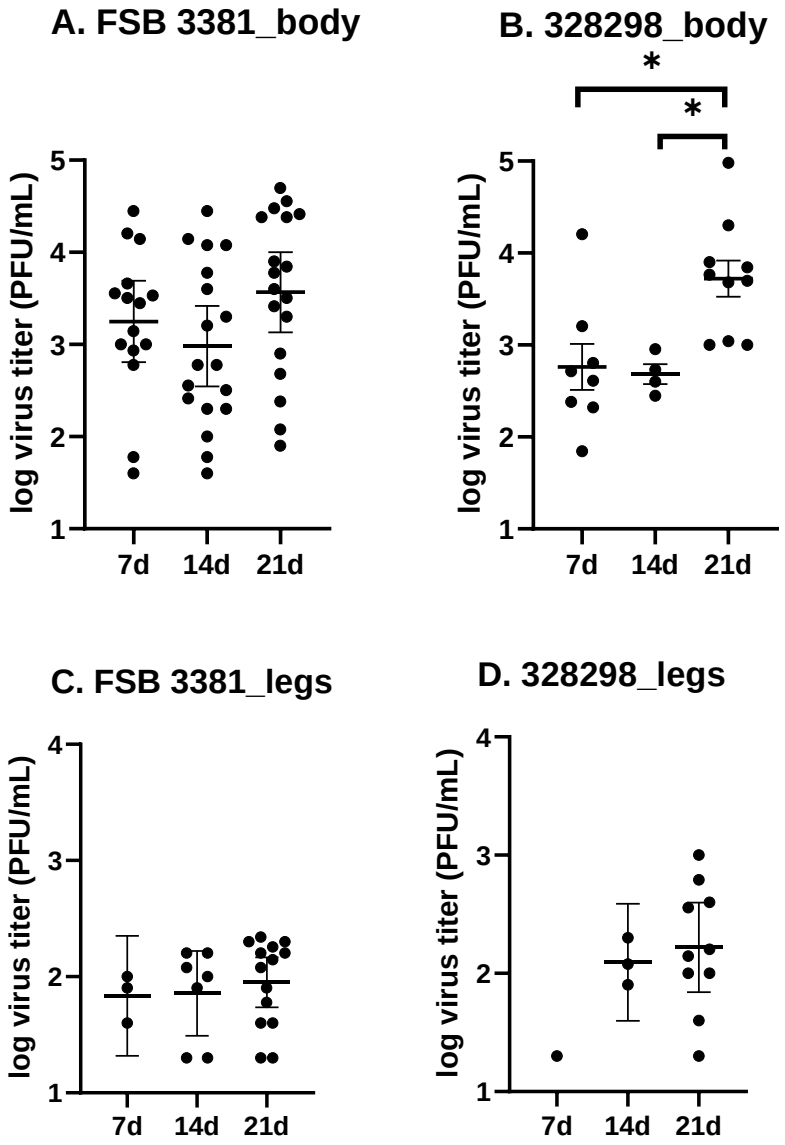


Figure 3.26. DENV-2 replication of the DENV 2 (FSB 3381) in body (**A**) and legs (**C**) or replication of DENV-2 (328298) in body (**B**) and legs (**D**) of El Paso *Ae. aegypti* at 7, 14, and 21 days after ingesting 4 log $_{10}$ PFU/ mL of virus.

Viral replication in Rio Grande *Ae aegypti* mosquito's bodies after ingesting 4 log $_{10}$ PFU/mL DENV-2 (FBS 3381) varied through time and had a mean body titer of 3.256 log $_{10}$, 2.677 log $_{10}$, 3.554 log $_{10}$ PFU/mL at 7, 14, and 21dpi, respectively (**Figure 3.27**). However, the

mean viral titer was not significantly different during the incubation period. *Ae. aegypti* mosquitoes that ingested DENV 2 (328298) strain had a similar mean body viral titer of 4.055 $\log_{10}$, 3.99 $\log_{10}$, 3.871 $\log_{10}$ PFU/mL at 7, 14, and 21dpi, respectively. However, the limited number of mosquitoes could have affected the results of statistical analysis. Finally, the viral titer in the *Ae. aegypti* mosquito legs infected with DENV-2 (FSB 3381) was 3.204 $\log_{10}$, 2.102 $\log_{10}$, and 2.040 $\log_{10}$ PFU/mL at 7, 14, and 21 dpi, respectively, while the viral titer in the legs of the mosquito infected with DENV-2 (328298) was 3.114 $\log_{10}$, 2.558 $\log_{10}$, 2.730 $\log_{10}$ PFU/mL at 7, 14, and 21 dpi, respectively.

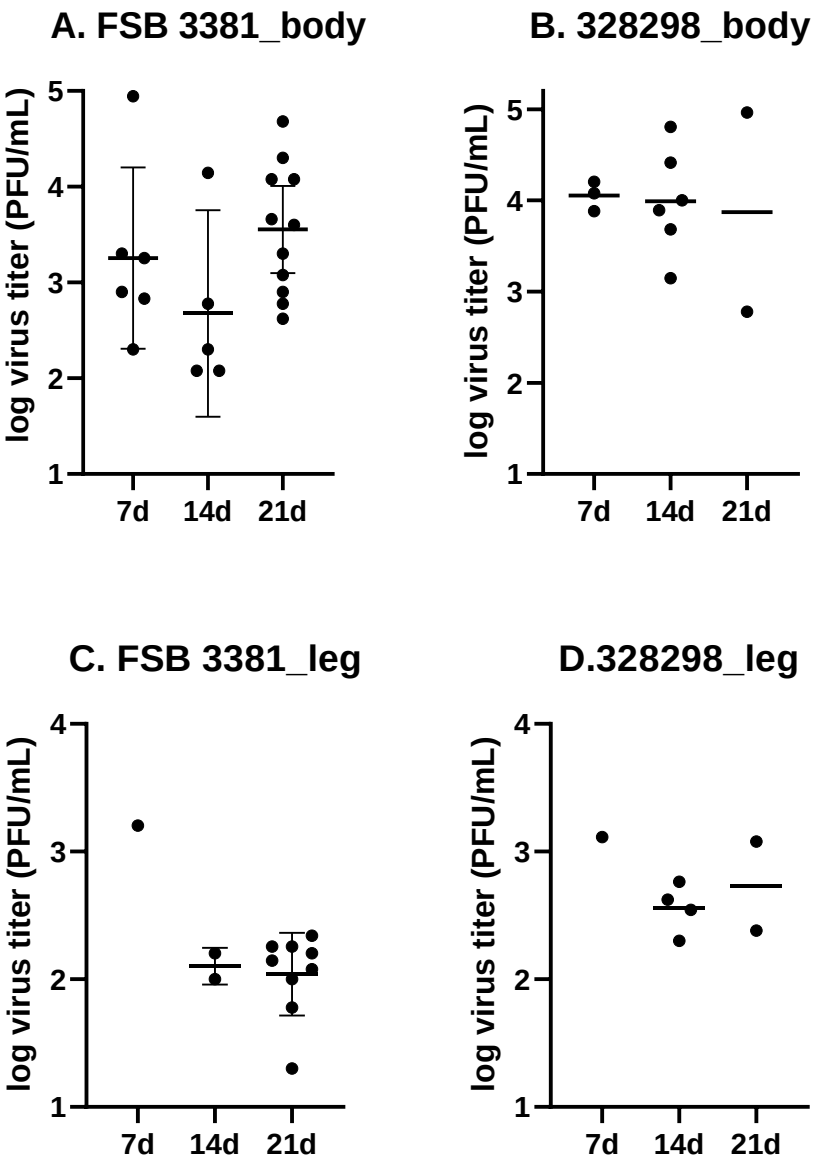


Figure 3.27. DENV-2 replication of the DENV 2 (FSB 3381) in body (**A**) and legs (**C**) or replication of DENV-2 (328298) in body (**B**) and legs (**D**) of Rio Grande *Ae. aegypti* at 7, 14, and 21 days after ingesting 4 $\log_{10}$ PFU/ mL of virus.

3.5. Evaluation of the effect of temperature on the vector competence of *Ae. aegypti* from the U.S. border region

3.5.1. Material and Methods

3.5.1.1. Standard rearing techniques of *Ae. aegypti*

Ae. aegypti mosquitos' strains (**Table 3.1.)** were reared in the UTEP Insectary as described in detail in the above section (Section 3.2.1.1)

3.5.1.2. Oral mosquito infections

Third (F3) *Ae. aegypti* generations from El Paso and Rio Grande were tested for DENV-2 oral infection as mentioned in the previous section (Section 3.2.1.4) with modifications in the incubation temperature and period. Briefly, a single viral dose of 6 $\log_{10}$ PFU/ mL of freshly propagated DENV-2 (FSB 3381) was mixed with of 2 mL defibrinated sheep blood (Colorado Serum Company, Denver, CO). An aliquot of each infectious blood meal was collected before and after feeding the mosquitoes to determine the infectious DENV-2 titer.

After mosquitoes ingested the virus-blood mixture, mosquitoes were cold anesthetized and only the fully engorged females were transferred to16 ounce mesh-top cardboard holding cups and fed with 10% sucrose in a chamber maintained at four different temperatures ranging between 20°C, 24°C, 28°C, and 32°C. Mosquitoes were kept at 14h: 10h light: dark cycle and 80% humidity. On days 5, 10, 15 and 20 p.i. batches of 3 to 7 individual mosquitoes were tested to determine the infection, dissemination, and transmission rates. Furthermore, to determine the amount of DENV-2 ingested by the mosquitoes, two to five engorged mosquitoes were stored individually immediately after they fed at -80°C for plaque assays.

3.5.1.3 Mosquito processing

Each mosquito was tested as follows: abdomen and thorax were tested to determine infection rates and the legs and wings were tested to determine disseminated infection rates; and saliva was tested to determine the transmission rates (Section 3.3.1.3).

3.5.1.4. Viral detection and quantification

Mosquito bodies, legs, and saliva were tested by plaque assays for virus in C6/36 cells and virus was identified by the IFAT as described previously (Section 3.2.1.6).

3.5.1.5. Statistical Analysis

Statistical analysis was performed as described in the previous section (Section 3.4.1.5). Briefly, the effect of temperature on the infectivity and disseminated infection rates was determined by using Fisher's exact analysis. Significant differences in the viral replication in body and legs of both *Ae. aegypti* mosquito strains affected by temperature were determined by using ANOVA. GraphPad Prism software 8.0 for statistical analysis.

3.5.2. Results

3.5.2.1. Temperature affected the DENV-2 infectivity and disseminated infection rates in Rio *Ae. aegypti*

Rio Grande *Ae. aegypti* mosquitoes that ingested DENV-2 (FSB 3381) were maintained at a different temperature ranging from 20°C to 32°C (**Figure 3.28**). At 20°C, infectivity rates ranged from 25% (1/4) to 100% (6/6). However, disseminated infection was not detected during the 20 days of incubation period. At 24°C, infectivity rates ranged from 66.7% (2/3) to 100% (5/5), while viral disseminated infection rates were detected at 15 dpi (50%, 2/4) only. At 28°C, infectivity rates ranged from 50% (2/4) to 100% (5/5) while the disseminated infection rates were 33% (1/3) at 5 dpi and reached 100% (4/4) at 10 dpi. Finally, at 32°C, infectivity rates ranged from 75% (3/4) to 100% (4/4), while the disseminated infection rates increased through the incubation period, as 50% (2/4) at 5 dpi, 66.7% (2/3) at 10 dpi, and 100% (4/4) at 15 and 20 dpi.

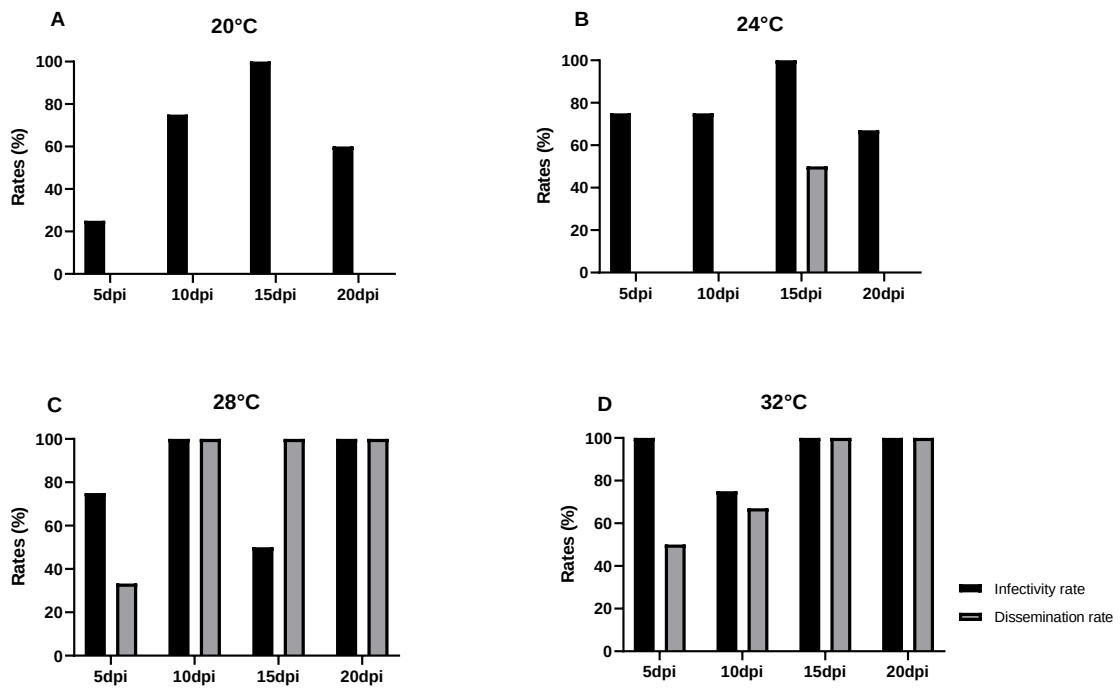


Figure 3.28. Infectivity and disseminated infection rates of Rio Grande *Ae. aegypti* after ingesting 5.5 log $_{10}$ PFU/ mL of DENV-2 (FSB 3381) and maintained at 20°C (**A**), 24°C (**B**), 28°C (**C**) or 32°C (**D**).

Virus was not detected in the saliva of any of the Rio Grande *Ae. aegypti* mosquitoes that had disseminated infection with DENV-2, or a transmission rate of 0%.

The overall DENV-2 infectivity rate of Rio Grande *Ae. aegypti* mosquitoes at 20°C, 24°C, 28°C and 32°C was 64.7% (11/17), 80% (12/15), 82.35% (14/17), and 93.75% (15/16), respectively (**Figure 3.29**). A comparison of the infectivity rates at 20°C vs 32°C showed the largest difference (64.7% vs 93.75%) and with a certain trend toward significance ($p=0.081$). Furthermore, no significant differences ($p>0.05$) were found between the infectivity rates at incubation temperatures higher or equal to 24°C. DENV-2 disseminated infection rates in Rio Grande *Ae. aegypti* mosquitoes were null (0%, 0/11) or low (16.67%, 2/12) during an extrinsic incubation temperature of 20°C and 24°C, respectively (**Figure 3.29**). Higher temperatures of

incubation such as 28°C and 32°C caused a DENV-2 disseminated infection rate of 85.71% (12/14) and 80% (12/15) respectively. Temperature of incubation at 20°C and 24°C significantly decreased the disseminated infection rates in comparison to the disseminated infection rates at 28°C and 32°C. No significant differences in the disseminated infection rates were observed at 28°C vs 32°C.

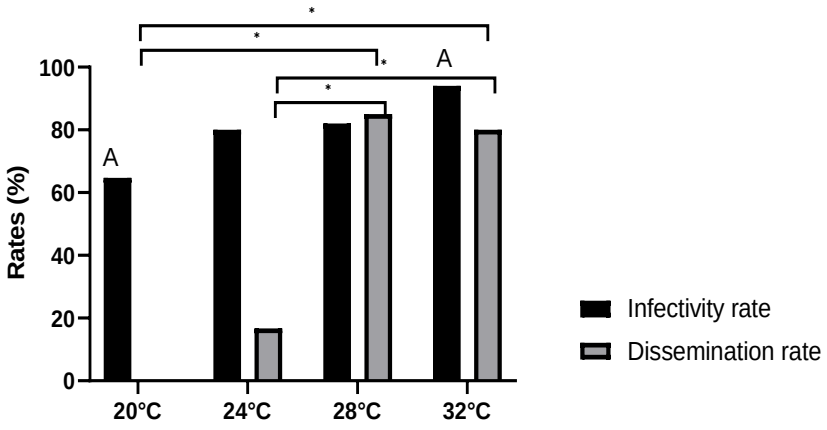


Figure 3.29. Overall infectivity and disseminated infection rates of Rio Grande *Ae. aegypti* after ingesting 5.8 log $_{10}$ PFU/ mL of DENV2 (FSB 3381) and maintained at 20°C, 24°C, 28°C, or 32°C. A= nearly significant differences (p=0.081)

3.5.2.2. Viral replication in Rio Grande *Ae. aegypti* mosquito tissues increased with temperature

Mean viral titer in Rio Grande *Ae. aegypti* bodies increased with an increased extrinsic incubation temperature (**Figure 3.30A**). Mean viral titer at 20°C and 24°C ranged from 2.45 to 3.1 log $_{10}$ PFU/mL and 2.85 to 3.78 log $_{10}$ PFU/mL respectively. In contrast, high means viral titers were detected at 28°C and 32°C ranging from 3.67 to 4.57 log $_{10}$ PFU/mL and 4.26 to 4.61 log $_{10}$ PFU/mL, respectively.

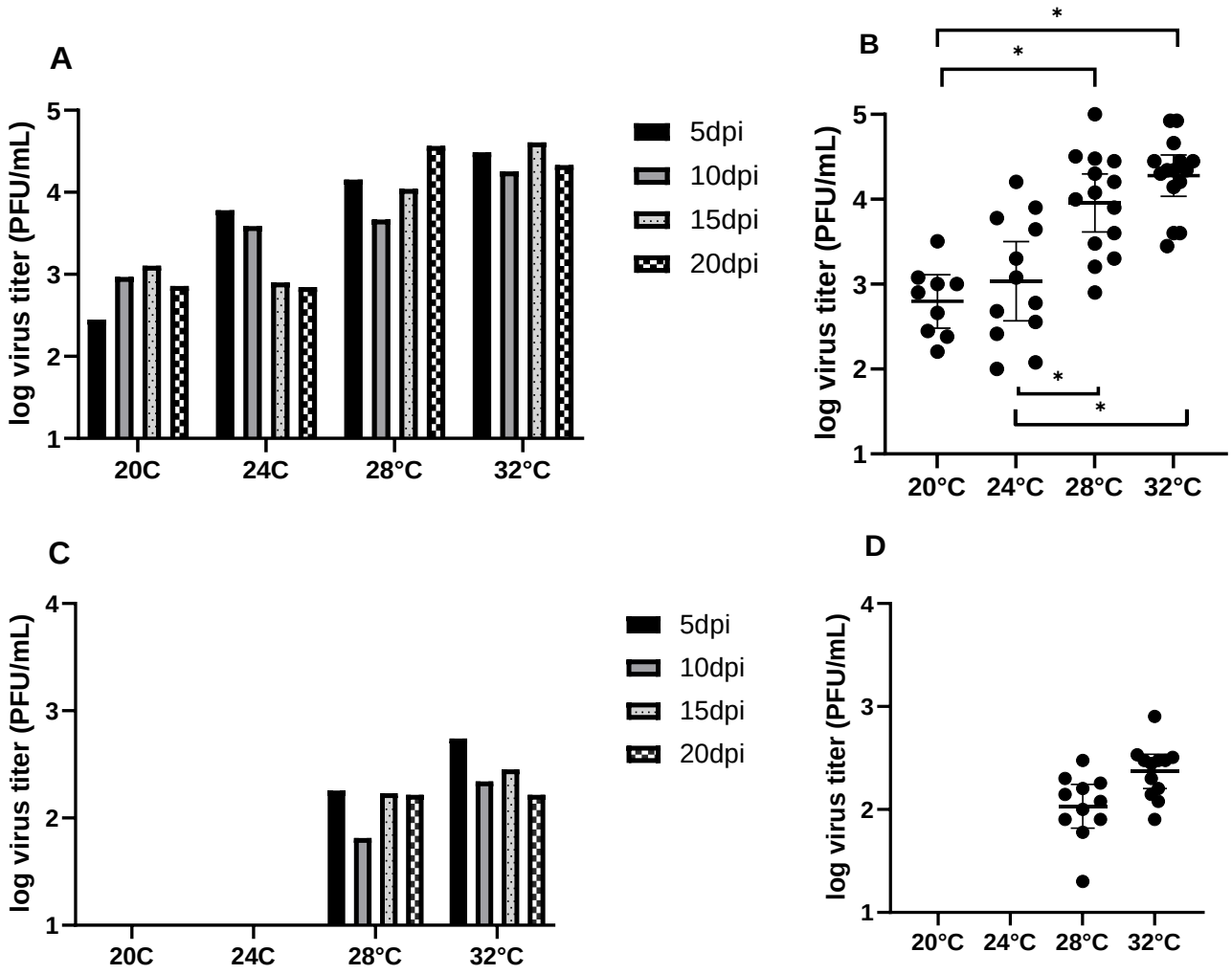


Figure 3.30. DENV-2 replication in Rio Grande *Ae. aegypti* mosquitoes maintained at 20°C, 24°C, 28°C, or 32°C during 20 days of incubation. Upper panel: **A**. Mean body viral titer. **B**. Distribution of body viral titer from 20°C to 32°C. Lower panel: **C**. Mean viral titers in the legs. **D**. Distribution of viral titers in the leg at incubation temperatures of 20°C to 32°C.

Overall, body viral titers increased significatively ($p<0.05$) with higher temperatures (**Figure 3.30B**), reaching the mean highest titer of 4.28 $\log_{10}$ PFU/ mL at 32°C, in comparison to lower viral titers determined at 20°C and 24°C of 2.8 $\log_{10}$ and 3.04 $\log_{10}$ PFU/mL.

Viral titers in legs were not detected at 20°C and 24°C, while at 28°C and 32°C titers ranged from 1.78 to 2.48 log $_{10}$ PFU/mL and 1.9 to 2.9 log $_{10}$ PFU/mL, respectively (**Figure 3.30C**. Furthermore, even though viral titer in the legs at 32°C was slightly higher than at 28°C (**Figure 3.30D**), viral titer in the legs at 28°C and 32°C did not differ significatively ($p>0.05$).

3.5.2.3. Temperature affected the DENV-2 disseminated infection rates in El Paso *Ae. aegypti* mosquitoes

El Paso *Ae. aegypti* mosquitoes ingested 6.1 log $_{10}$ PFU of DENV-2 (FSB 3381) and maintained at 20 °C to 32° C for 20 days (**Figure 3.31**). Infectivity rates were not affected by any of the extrinsic incubation temperatures ranging from 24°C to 32° C displaying rates of infection from 83% (5/6) to 100% (6/6) at different times of incubation. On the other hand, disseminated infection rates at 24°C, increased through the incubation time ranging from 0% (0/5) to 100% (6/6), while disseminated infection rates at 28°C and 32°C were similar and ranged from 80% (4/5) to 100% (6/6). At 20°C, no samples were collected at 5 and 10 dpi, but the infectivity rates reached 100% (6/6) at 15 dpi, while disseminated infection rates were 66.7% (4/6) and 100% (3/3) at 15 dpi and 20 dpi, respectively). Finally, no virus was detected in any of the mosquito saliva samples that had disseminated infection in the legs.

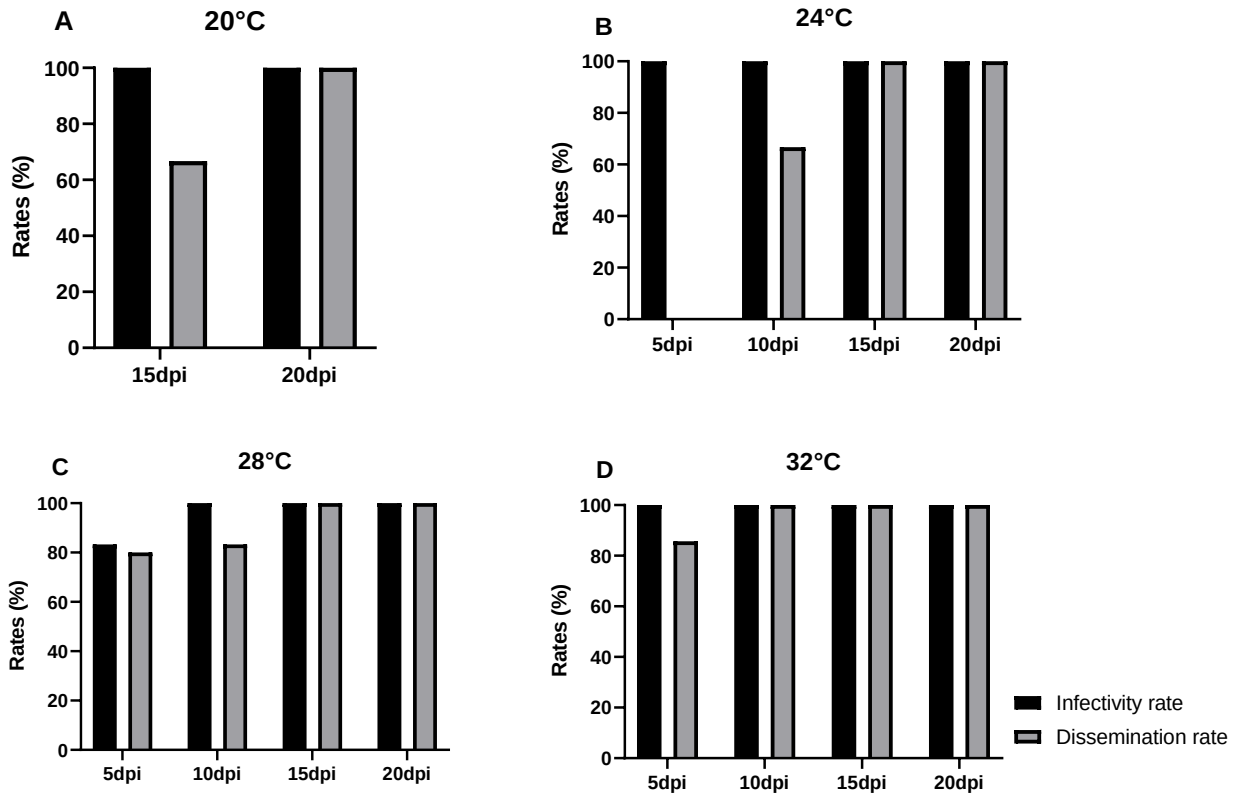


Figure 3.31. Infectivity and disseminated infection rates of El Paso *Ae. aegypti* after ingesting DENV-2 (FSB 3381) containing 6.1 $\log_{10}$ PFU/ mL and maintained at 20°C (**A**), 24°C (**B**), 28°C (**C**) or 32°C(**D**).

The overall infectivity rates at 24°C, 28°C, and 32°C were 100% (25/25), 96% (24/25) and 100% (27/27) respectively, while the disseminated infection rates were 72% (18/25), 91.67% (22/24), and 100% (27/27), respectively (**Figure 3.32**). No significant differences were noted in the overall infectivity rates from 24°C to 32°C for El Paso *Ae. aegypti* mosquitoes. However, disseminated infection rates were significantly reduced ($p<0.05$) at a lower temperature (E.g., 24°C) in comparison to the highest temperature of incubation (32°C).

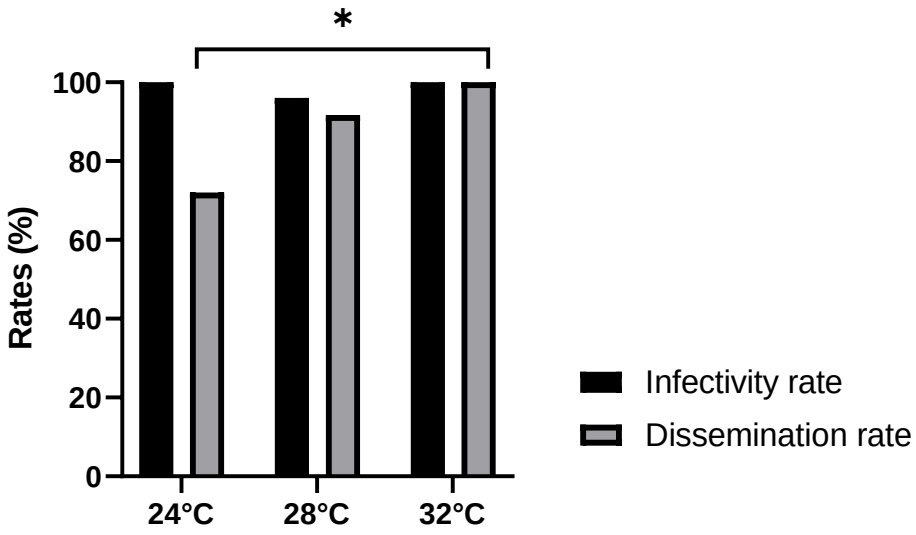


Figure 3.32. Overall infectivity and disseminated infection rates of El Paso *Ae. aegypti* after ingesting 6.1 log $_{10}$ PFU/ mL of DENV-2 and maintained at 24°C, 28°C or 32°C.

3.5.2.4. Viral replication in El Paso *Ae. aegypti* mosquito tissues increased with temperature

Mean viral titer in El Paso *Ae. aegypti* bodies at 15 dpi and 20 dpi maintained at 20°C were 2.85 log $_{10}$ PFU/mL and 2.97 log $_{10}$ PFU/mL, respectively (**Figure 3.33A)**. At 24°C, body titers ranged from 3.4 log to 4.03 log $_{10}$ PFU/mL while at 28°C, titers ranged from 3.38 log $_{10}$ to 4.56 log $_{10}$ PFU/mL. At 32°C, body titers ranged from 3.96 log $_{10}$ to 4.81log $_{10}$ PFU/mL. Significant differences ($p<0.05$) in the body titer were associated with temperature (**Figure 3.33B**). The mean viral titer of mosquito bodies at low temperatures such as 20°C and 24°C was and 2.74 log $_{10}$ and 3.45 log $_{10}$ PFU/mL in comparison to higher temperatures of 28°C and 32°C that were 4.13 log $_{10}$ and 4.25 log $_{10}$ PFU/mL, respectively. Overall, the mean body titers at 20°C and 24°C were significantly different ($p<0.05$) from any mean body titer subjected to other temperatures of incubation.

The mean viral titer in the leg through time are displayed in **Figure 3.33C**. Overall, viral titer in legs maintained at 20°C (1.64 log $_{10}$ PFU/mL) was significantly lower ($p<0.05$) than viral titer in the legs at 28°C and 32°C (2.31 log $_{10}$ and 2.18 log $_{10}$ PFU/mL) (**Figure 3.33D**). No significant differences in the leg viral titer were determined between the two low temperatures (20°C and 24°C) and the two high temperatures (28°C and 32°C).

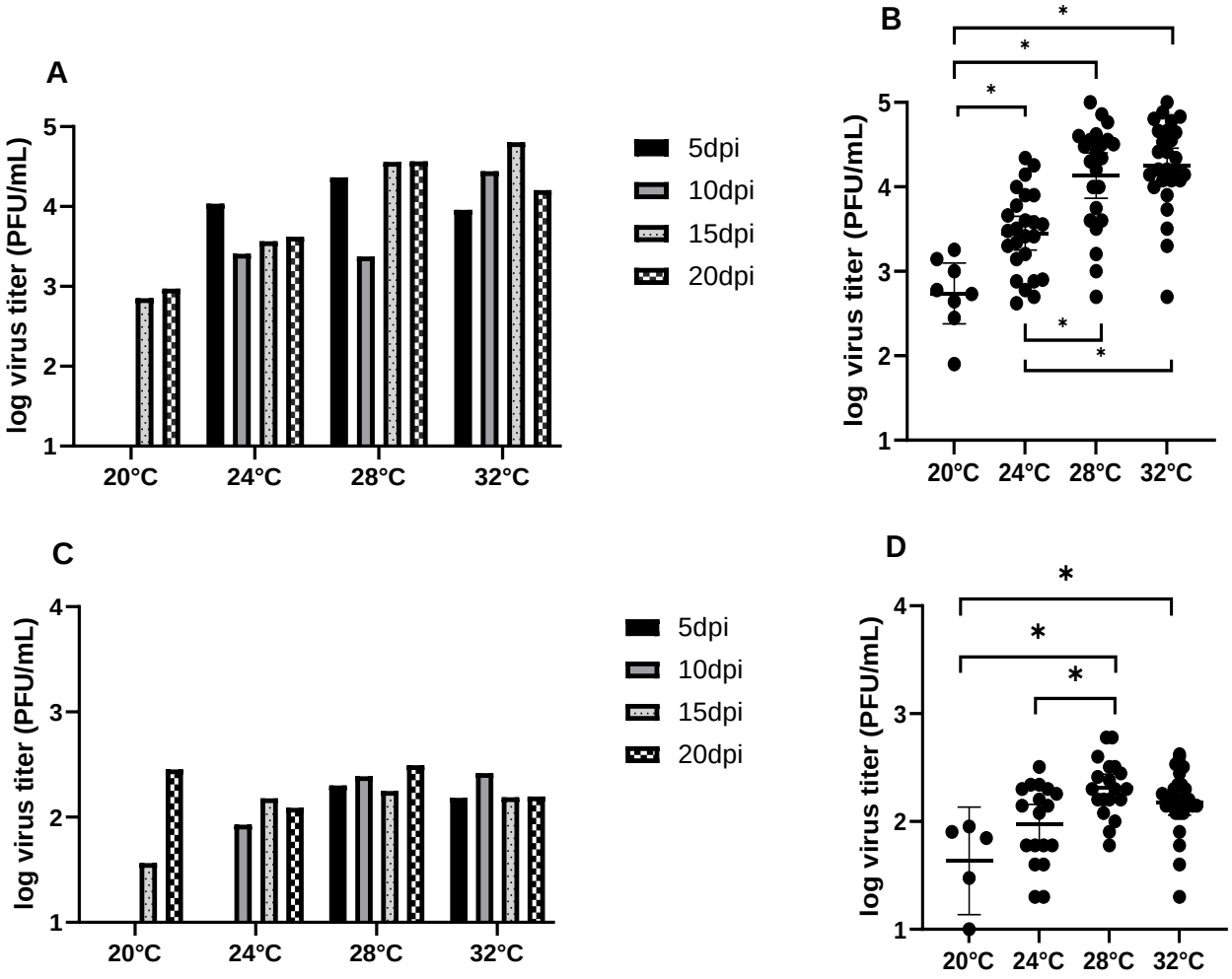


Figure 3.33. DENV-2 replication in El Paso *Ae. aegypti* mosquitoes maintained at 20°C, 24°C, 28°C or 32°C during 20 days of incubation. Upper panel: **A**. Mean body viral titer from 5 to 20 dpi. **B**. Distribution of viral titer in the body ranged from 20°C to 32°C. Lower panel: **C**. Mean viral titer in the legs **D**. Distribution of viral titers in leg at20°C to 32°C.

3.6. Discussion

3.6.1. Susceptibility of El Paso and Rio Grande *Ae. aegypti* mosquitoes to infection with DENV-2

This study was the first to evaluate the susceptibility and vector competence of *Ae. aegypti* mosquitoes from the Texas U.S. border region such as El Paso and Brownsville (Rio Grande Valley) to DENV. The first aim was to evaluate the susceptibility of *Ae. aegypti* to infection with DENV-2 including both *Ae. aegypti* strains after ingesting DENV-2 American genotype (382898) isolated from the Mexican border city of Reynosa in 1995, the susceptibility to viral infection or infectivity rate were higher than 70% at 7 to 14 dpi. A subsequent study, using a different DENV-2 Asian/American genotype (FSB 3381) isolated from South America (Bolivia), also showed that the viral infection susceptibility was >70% for both El Paso and Rio Grande *Ae. aegypti* strains with no significant differences in the infectivity rates.

Further analysis of the mean amount of infectious particle or PFU in the whole mosquito body through the 14 days extrinsic incubation period, indicated 10 times increase of ~ 4 $\log_{10}$ PFU in comparison to the initial amount of ~3 $\log_{10}$ PFU at time 0, immediately after ingesting virus, the virus began to replicate in the *Ae. aegypti* mosquitoes from the U.S. border regions. A similar mean viral titers (ranging from 3 to 4 $\log_{10}$ PFU) in orally infected *Ae. aegypti* mosquitoes with Asian /American DENV-2 (JAM 1409) from South Mexico (Chetumal) for 14 days incubation period was determined previously (Salazar et al., 2007), further supporting the consistent increase of DENV-2 titer in the U.S. border *Ae. aegypti* infected mosquito bodies.

3.6.2. Vector competence of El Paso and Rio Grande *Ae. aegypti* to DENV strains

Based on the observation that El Paso and Rio Grande *Ae. aegypti* mosquitoes were susceptible to infection with DENV-2 (FSB 3381) and DENV -2 (328298), the next aim was to determine their vector competence for DENV-2 strains. In addition to FSB 3381 and 328298 strains, a 16681 strain (Asian Genotype) was also included in the vector competence evaluation. Initially, the identity of the DENV-2 strains: 328298, 16681, and FSB 3381 used in this study was confirmed by sequencing of the E gene. Furthermore, a Maximum likelihood Phylogenetic analysis of the E gene grouped each 328298, 16681, and FSB 3381 strains into corresponding American, Asian 1, and Asian/ American genotypes respectively (**Figure 3.2**). Previous vector competence studies have also confirmed the identity and genetic background of the dengue strains by sequencing and performing phylogenetic studies (Armstrong & Rico-Hesse, 2003; Cologna et al., 2005).

El Paso and Rio Grande *Ae. aegypti* had varied infection rates according to the viral strain. Both *Ae. aegypti* strains had infection rate ranging from 60% to 100% for DENV 2 (FSB 3381) and DENV-2 (328298) strains with no significant differences. Infection rates for DENV 2 (FSB 3381) and 328298 were associated with the incubation time in *Ae. aegypti* from Rio Grande, while for El Paso *Ae. Aegypti*, association between incubation times and infectivity rates was nearly significant. By contrast, the infection rate for the DENV-2 (16681) strain was significantly low (4-13%) in comparison to DENV 2 (FSB 3381) and DENV-2 (328298) in both *Ae. aegypti* strains through the incubation period. Overall, these observations for the two DENV-2 strains (FSB 3381 and 328298) infection in both *Ae. aegypti* strains, indicated that these DENV-2 viral strains did not differ significantly in establishing initial viral infection in the *Ae. aegypti* mosquitoes. Furthermore, no significant differences were observed in the in vitro

replication between the two DENV-2 strains in C6/36 cells during the 10 days incubation period (**Appendix 1**). The low infection rate (4 to 13%) for DENV- 2 (16681) strain reported in this study for both *Ae. aegypti* strains was also reported in other vector competence studies using DENV-2 Asian genotypes (Gloria-Soria et al., 2017). Briefly, DENV-2 Hanoi (6H) and DENV-2 HCM (434S) strains belonging to the Asian genotype had infectivity rates of 9.1% and 13% respectively in *Ae. aegypti* mosquitos from Vietnam (Gloria-Soria et al., 2017). The low susceptibility infection rate for DENV-2 (16681) in *Ae. aegypti* differs from the high in vitro 16681 replication in C6/36 cells (**Appendix 1**), indicating that an in vitro cell system might not accurately represent a more complex in vivo system, where other mechanisms and mosquito tissue barriers are implicated in the infectivity rate.

On the other hand, there have not been studies to identify a DENV genetic determinant that can affect viral infectivity in *Ae. aegypti* mosquitoes. However, a recent study identified a single amino acid change (R120) on the domain II of the DENV-2 (16681) E protein that decreased the infectivity rate in the *Ae. aegypti* midgut (Erb et al., 2022). Interestingly, the 16681-strain used in this study also carries this positive amino acid (R120), while the FSB 3381 and 328298 strains have an uncharged amino acid (T120) that supports the low infectivity rate of 16681 reported in this study. Furthermore, the presence of positively charged amino acids in the DII reduced the mosquito midgut infection not only in this U.S. border *Ae. aegypti*, but also in a highly susceptible *Ae. aegypti* mosquitoes from Chetumal (Erb et al., 2022). It might be possible that due to high number of in vitro passages of the DENV-2 (16681) strain since the virus was isolated in 1964, have caused the apparition of the R120 in the E protein, as the R120 is absent in other contemporary DENV-2 strains.

Disseminated infection rates for DENV- 2 (FSB 3381) and DENV-2 (328298) strains in *Ae. aegypti* from El Paso and Rio Grande had a significant increase through the incubation period and reached the highest rate (80%-100%) at 14 dpi. No significant differences in the disseminated infection rates between both *Ae. aegypti* strains were observed and only a few *Ae. aegypti* mosquitoes from Rio Grande had disseminated infections with 16681, while no disseminated infection was observed in the El Paso *Ae. aegypti*. Furthermore, a low transmission rate (1.89%-2.78%) was found in El Paso and Rio Grande *Ae. aegypti* for DENV-2 (FSB 3381) and DENV-2 (328298) strains. Another vector competence study using *Ae. aegypti* from McAllen, Texas obtained a lower dissemination rate (50%) than our study for three other DENV-2 strains belonging to the American genotype (IQT 2913, Ven2, and 131 strains) at 14 dpi (Anderson & Rico-Hesse, 2006). An explanation for this difference may be due to a different genetic background of *Ae. aegypti* (McAllen vs Brownsville), time of collection of field mosquitoes (2001 vs 2018), viral exposure dose (RNA genome equivalent vs PFU), and the DENV-2 (328298)-strain used in this study. Viral dissemination is a critical step to determine vector competence and is dependent on the period needed for the virus to reach the salivary glands from the midgut. Furthermore, as reported in this study, disseminated infection rates of the DENV-2 American genotypes increased through the incubation period (Anderson & Rico-Hesse, 2006).

Interestingly, *Ae. aegypti* mosquitoes from Houston, Texas were tested for susceptibility to DENV-2 (JM 1409) Asian/America strain and displayed a 60% and 65% of infectivity and dissemination rate, respectively (Bennett et al., 2002) in comparison to the slightly higher infectivity and disseminated infection rates of 70% and 80%, respectively for a DENV-2 (323898) and DENV-2 (FSB 3381) strains in El Paso and Rio Grande *Ae. aegypti* reported in this

study, indicating that, *Ae. aegypti* mosquitoes from Texas have been previously reported to be DENV-2 susceptible. However, the extensive 25 number of passages of DENV-2 (JM 1409) in C6/36 cells could have affected the susceptibility and the data might not accurately represent a natural dengue mosquito infection as it was performed in this study, where the DENV-2 strains used (FSB 3381 and 328298) had a low number of passages in vitro.

The low DENV-2 transmission rate in both *Ae. aegypti* strains from El Paso and Rio Grande, even though a high disseminated infection rate was obtained, suggested that a salivary gland barrier may have prevented the release of the virus into the saliva. This barrier could have prevented DENV-2 infection of the salivary glands (salivary gland infection barrier) or have prevented the release of DENV-2 into the salivary lumen that goes to the saliva (salivary gland escape barrier). Since the salivary glands were not tested separately in this study, we could not determine the effect of salivary infection or salivary gland escape barriers. However, previous studies suggested a salivary gland barrier for DENV in *Ae. aegypti* mosquitoes from Florida where also occurred a lack of correspondence in the disseminated infection and transmission rates (Richards et al., 2012). Recently, a genetic basis for salivary gland infection and for an escape gland barrier was reported for the DENV-2 American (Qro94) and Asian (C93) genotypes that were studied in a freshly colonized strain of *Ae. aegypti* from Chiapas (Mexico) (Sanchez-Vargas et al., 2021) . Briefly, a low salivary gland infection barrier (0.8-5.5%) but an elevated salivary gland escape barrier (73-89%) were reported for the American and Asian DENV-2 genotypes, based on the high rates of infected salivary glands but low presence of virus in the saliva, especially in the American genotype (11% transmission rate) while in the Asian genotype was 27%. (Sanchez-Vargas et al., 2021), indicated that the salivary gland barrier traits in the mosquitoes is dependent on the viral genotype or the viral growth rate in the salivary glands. If

this is the case, a possible salivary escape barrier could explain the low (~2%-3%) transmission rates observed in this study for the U.S. border *Ae. aegypti*, not only for the DENV-2 (328298) American genotype (closely related to Qro94 strain), but and possibly for the FSB 3381 DENV-2 Asian/American genotype.

Variation in the vector competence has been linked to genetic interactions between the arboviral genotypes and *Ae. aegypti* mosquitoes, also known as gene by gene (G x G) interactions, (Lambrechts, 2011; Sanchez-Vargas et al., 2021). The genetic basis of these interactions is not entirely understood, and future studies are still needed. In addition, the mosquito innate immunity genes may have affected viral replication when the viral titer was low, therefore when the infecting virus replicated rapidly, the virus could overcome the innate immune response as could have occurred with 6 $\log_{10}$ PFU/mL viral dose. As in this study, low generations of mosquitoes from the U.S. border region were used in order to represent a nature population, where innate immune genes were present.

Another reason for the low transmission rate was because of the forced salivation technique. The procedure for forcing a mosquito to salivate is performed by placing the proboscis into a capillary tube containing mineral oil or pipette tip and subsequent harvesting of the saliva for testing for virus. The forced salivation technique has been used as an option for measuring viral transmission instead of allowing mosquitoes to feed on a susceptible animal as a method for determining transmission rates. However, the technique is laborious, a high level of skill is needed, and requires standardization, and validation of the procedures and components, such as collection diluent (mineral oil, blood, fetal bovine serum, sugar), mosquito immobilization procedures (CO_2, cold, triethylamine) between others, Interestingly, a recent study found significantly higher positive DENV saliva rates for Florida *Ae. aegypti* mosquitoes

using blood rather than mineral oil for collecting the saliva (DENV-4 Haitian strain transmission rates went from 7% to 30% and for DENV-4 Philippines strain went from 2.5% to 23%) (Stephenson et al., 2021), suggesting that using blood as a saliva collection method might be a more realistic proxy for DENV transmission to humans. In our study, we collected the mosquito saliva in mineral oil, and then saliva was tested in C6/36 cells for viral infectivity, and cells were tested for IFAT to DENV. Thus, the 2-3% saliva-positive rate represented infective DENV, and rates were similar to the positive rate mentioned above using mineral oil. The sensitivity of our infectivity assays ranged from 0.1 to 1.5 PFU and the C6/36 cells inoculated with the saliva suspensions were also tested for DENV RT-PCR, which confirmed the 2-3% positive rate of the samples. However, it cannot be ruled out that the viral titers in saliva were under the limit of detection, possibly caused by other factors as vector-virus genotype pairing or mosquito re-ingestion of the saliva during the forced salivation (Miller et al., 2021). Finally, an underestimation of the transmission potential using forced salivation in *Ae. aegypti* for several arboviruses has been reported (Gloria-Soria et al., 2022), indicating that the use of mosquito legs and salivary glands might be a more accurate predictor (proxy) of transmission than saliva. If we extrapolate this assertion, the potential DENV transmission by these two *Ae. aegypti* strains would be highly significant (~80%-90%). However, as this is a pioneer study of vector competence of *Ae. aegypti* from the U.S. border for certain DENV strains (G x G interactions), multiple approaches are needed to determine the DENV transmission and a possible salivary gland barrier in these *Ae. aegypti* strains. Future studies using a proper animal model for DENV transmission such as the AG 129 immunocompromised mice (Baldon et al., 2022) (double knockout lacking alpha/beta and gamma interferon receptors) could possibly prove to be a better method demonstrating DENV-2 transmission by *Ae. aegypti* mosquitoes.

Midgut mosquito microbiota as bacteria or Insect specific virus (ISV) can also interfere with the vector competence of certain Flaviviruses (Blitvich & Firth, 2015). In the Rio Grande *Ae. aegypti* strain, a Cell Fusing agent virus (CFAV) was detected in some male and females' mosquitoes from the F3 generation used for the vector competence experiment. The role of CFAV has been reported to interact with DENV replication in vitro or affect DENV dissemination titers in *Ae. aegypti* head tissues (Baidaliuk et al., 2019; Zhang et al., 2017). In our study, mosquito legs were used to determine viral dissemination in Rio Grande *Ae. aegypti* and reached almost 100% with no significant differences in the viral titer leg, suggesting that any other possible mechanism mentioned above might be involved. The presence of CFAV in the Rio Grande *Ae. aegypti* strain supports the finding of CFAV in field *Ae. aegypti* mosquitoes from Rio Grande Valley (McAllen, Brownsville) and San Antonio, Texas, where the infectivity rate (per 1000 individuals) was 56.0 and 166.8 respectively in 2018 (Martin et al., 2020). By contrast, no ISFV was detected in El Paso *Ae. aegypti* strain used in this study and in another field collected and recently colonized *Ae. Aegypti* from El Paso. Further studies using the CFAV isolated from Rio Grande *Ae. aegypti* in this study would provide information of its role in the vector competence of *Ae. aegypti* for DENV.

3.6.3. Viral dose role on the vector competence to DENV

Viral dose is critical for the initial infection that occurs in the mosquito midgut and is associated with the presence of the midgut infection barrier. Human viremia caused by DENV can range from 2 log to 7 log $_{10}$ PFU/mL (Shu et al., 2003), indicating the relevance of our study using 4 log $_{10}$ PFU/mL as a dose of virus for infecting *Ae. aegypti* mosquitoes. Unlike the similar infectivity rates for DENV-2 (FSB 3381) and DENV-2 (328298) in both U.S. *Ae. aegypti* strains after ingesting 6 log $_{10}$ PFU/mL viral dose, significant differences were observed between the

DENV-2 strains and *Ae. aegypti* strains after ingesting a lower dose of 4 log $_{10}$ PFU/mL. Infectivity rates using the DENV 2 (328298) were reduced from 60%-100% to 10%-50% for both *Ae. aegypti* strains, while the infectivity rates to DENV-2(FSB 3381) were not affected in El Paso mosquitoes (from 60-90% to 75-85%) but were significantly reduced in Rio Grande mosquitoes (from 76-96% to 25-50%). This differential DENV infection susceptibility was also detected in two field *Ae. aegypti* exposed to low infectious doses (median 4.74 log PFU) of DENV-2 strains in Thailand (Pongsiri et al., 2014). Furthermore, the DENV-2 (16681) strain did not infect any of the U.S. border *Ae. aegypti* strains (0%), supporting its inability to infect the *Ae. aegypti* midgut as occurred with a 6-log $_{10}$ PFU/mL viral dose (discussed above) which could have helped to slightly bypass the midgut barrier and caused the low infectivity rate of 4-13%. Interestingly, the mean viral titer immediately after the 4 log $_{10}$ of DENV-2 was ingested by the U.S. border *Ae. aegypti* mosquitoes was ~ 1 log $_{10}$ PFU/mosquito, which titer significant increased to 3.5 log $_{10}$ (mean mosquito body) in DENV-2 (FSB 3381) and DENV-2 (328298) orally exposed mosquitoes during the 7-21 days incubation period, indicating a successful viral replication of more than 100-fold (2 log $_{10}$).

The 50% mosquito infectious dose (MID_{50}) refers to the amount of virus that infect 50% of mosquitoes and it is epidemiologically important for understanding the dengue transmission when human viremias are not high (Gubler et al., 1981). Based on the infectivity rates in our study, it can be estimated that the MID_{50} for DENV-2 (16681) MID_{50} was higher than 6 log $_{10}$ PFU/mL for both U.S. border *Ae. aegypti* strains, while the DENV-2 (328298) MID_{50} was ~5 log $_{10}$ PFU/ml. Interestingly, DENV-2 (FSB 3381) MID_{50} differed in both U.S. *Ae. aegypti* strains, being ~ 3 log $_{10}$ PFU/mL and ~5 log $_{10}$ PFU/mL in El Paso and Rio Grande *Ae. aegypti* strains respectively, indicating its increased susceptibility of this contemporary DENV genotype in El

Paso *Ae. aegypti*. Another study using F3 generations *Ae. aegypti* from Vietnam reported a DENV-2 Asian genotype MID_{50} of 4.43 $\log_{10}$ PFU/mL after blood-feeding mosquitoes on viremic humans (Nguyen et al., 2013) . By contrast, other studies determined an elevated MID_{50} ranging from 6.5 to 7.4 $\log_{10}$ PFU/mL for a DENV-2 Asian/American genotype (Nicaragua isolate) in *Ae. aegypti* from Argentina (Ciota et al., 2018). Overall, a low MID_{50} translates to 1) a high chance for infecting mosquitoes from mild or asymptomatic dengue patients and 2) a longer window period for a mosquito to get infected from a low viremic patient during the disease resolution. Now, even though the low viral dose is direct associated with low infectivity rates and could affect the transmission potential as less infected mosquitoes might be able to transmit DENV, it needs to be considered that other external factors, such as extrinsic incubation period, temperature, and host immunity between others, are effective in the DENV transmission.

On the other hand, the highest disseminated infection rates in both *Ae. aegypti* strains were delayed after ingesting the 4-$\log_{10}$ PFU/mL viral dose (21 dpi) in comparison to the 6-$\log_{10}$ PFU/mL viral dose (14 dpi). However, viral replication in the mosquito body was able to reach similar titers, as occurred with the higher exposure dose, at a later incubation time, indicating that once viral replication was established in the infected mosquito bodies, viral disseminated infection trended to increase through time. Finally, a low transmission (1/13, 7.69%) or null (0%) transmission rate to DENV-2 (FSB 3381) occurred in Rio Grande and El Paso *Ae. aegypti*, respectively and might be due to the reasons discussed above.

3.6.4. Effect of temperature in vector competence to DENV for El Paso and Rio Grande *Ae. aegypti* mosquitoes

Temperature is an external factor that affects the vector competence of *Ae. aegypti* for DENV (Watts et al., 1986). As previously determined that El Paso and Rio Grande *Ae. aegypti*

were susceptible and supported viral disseminated infection of DENV-2 (FSB 3381) and DENV-2 (328298) strains, it was unknown if the temperature had an effect on the vector competence. The effect of temperature on the vector competence of DENV-2 (FSB 3381) strain was evaluated in both *Ae. aegypti* strains, as this strain belongs to a contemporary DENV-2 Asian/American genotype currently circulating in the Americas (de Jesus et al., 2020; Hernández-García et al., 2020), highlighting its epidemiological importance.

On El Paso and Rio Grande *Ae. aegypti*, the infectivity rates for DENV-2 (FSB 3381) increased with temperature (60%-100%), but the increase was not significant, while the replication of the virus increased significantly in the mosquito body in association with an increases in temperatures (28°C and 32°C), being the highest mean titer at 32°C (4.3 $\log_{10}$ PFU/mL) in comparison to the mean titer (2.8 $\log_{10}$ PFU/mL) at 20°C. This result indicated that DENV-2 (FSB 3381) viral replication is slow at low temperatures, but it is still enough to sustain mosquito body infection through the 20 days incubation period. The ability of DENV to replicate at low temperature is supported by the fact that the optimal temperature for initiation of viral RNA synthesis by the DENV RNA polymerase dependent of RNA occurs at 20°C and might be possible that viral RNA template adopts a conformational change for initiation at lower temperature facilitating the replication in *Ae. aegypti* mosquitoes (Ackermann & Padmanabhan, 2001). By contrast in other Flaviviruses as in ZIKV, the synthesis of ZIKV RNA Asian lineage is highly reduced at 20°C (Tesla et al., 2022).

Interestingly, disseminated infection and replication rates for DENV-2 (FSB 3381) strain in the legs of mosquitoes differed between El Paso and Rio Grande mosquitoes at low temperatures (20°C and 24°C) of incubation. While an overall low (16.67%) disseminated infection rate occurred in Rio *Ae. aegypti* at 24°C, a 64% disseminated infection rate occurred in

El Paso *Ae. aegypti*. Surprisingly, no disseminated infection occurred at 20°C in Rio *Ae. aegypti* through the 20 days of incubation, but a 60-100% disseminated infection occurred in El Paso *Ae. aegypti* at 15 to 20 dpi. These differences in the disseminated infection rates were supported by the lack of infective virus in Rio Grande mosquitos' legs (< 10PFU) in contrast to the 1.5-2.1 log $_{10}$ PFU/mL detected in El Paso mosquito legs. A possible explanation could be due to the presence of a midgut escape barrier in Rio Grande mosquitoes to DENV-2 (FSB 3381) more efficient at lower temperatures (20°C and 24°C) than a higher temperature (28°C and 32°C), and possibly caused by a strong mosquito immune response as the activation of JAK Stat or Toll Pathways (Ferreira et al., 2020). Therefore, our study detected an effect in the DENV disseminated infection rates that may have been caused by the mosquito strain and low temperatures. A study using *Ae. aegypti* from temperate regions from Argentina (La Plata) detected also high DENV-2 dissemination at extrinsic incubation temperatures ranging from 18°C to 23°C (mean = 20°C) (Ciota et al., 2018) as occurred with El Paso *Ae. aegypti* mosquitoes in this study. Another study reported an interaction between mosquito genetics and temperature affecting the DENV infectivity rates (disseminated infection rates were not evaluated) in *Ae. aegypti* mosquitoes from Hanoi (northern Vietnam, adapted to cold temperatures) vs *Ae. aegypti* from Ho Chi Minh (South Vietnam, subtropical temperature) (Gloria-Soria et al., 2017). This observation suggested that temperature adaptation in *Ae. aegypti* from Hanoi caused a strong mosquito immune response to DENV resulting in a lower DENV infection success in the midgut. The interaction between the mosquito genetics and temperature and their effect on the DENV at the different mosquito tissues and barriers is still unknown. Mosquito genetic studies of *Ae. aegypti* distributed along the US border could provide

information on their genetic variability and clarify the role of temperature on the activation of innate immune response in these *Ae. aegypti* mosquitoes.

As no infective DENV saliva was detected in the disseminated U.S. border *Ae. aegypti* mosquitoes, the extrinsic incubation period (EIP) could not be determined. However, based on the disseminated infection rates, an estimated EIP was extrapolated with caution. In the case of Rio Grande mosquitoes, the EIP was as early as 5 to 10 days at 28°C and 32°C, while in El Paso mosquitoes the EIP at low temperatures of 20°C and 24°C at 10 to 15 dpi, and a high temperature of 28°C and 32°C at 5 to 10 dpi.

Finally, *Ae. aegypti* population density studies in El Paso have shown the density peak from August to September in the last six years of mosquito surveillance (Vera & Watts 2022, Personal communication). This trend is associated with environmental factors such as seasonal rains (1.23-2.02 inch) during July to September and the elevated summer mean temperatures (25.6°C to 30.1°C) (ACIS, 2022). Based on the susceptibility and disseminated infection rates of the El Paso *Ae. aegypti* at different temperatures, the findings suggested that DENV 2 (FSB 3381) could replicate and disseminate in El Paso *Ae. aegypti* not only during warmer season of the year but also during cooler temperature (20°C) such as those that occur in October. Even though this feature could increase the potential of the El Paso mosquitoes to disseminate the virus, further studies using animal models are needed to evaluate the DENV transmission at low temperatures for El Paso *Ae aegypti*.

Chapter 4: Isolation And Characterization Of Dengue Virus From The Mexican/Us Border And Effects On The Vector Competence On U.S. Border *Ae. aegypti* Mosquitoes

4.1. Introduction

Dengue is the most important mosquito-borne viral human disease in the tropical and subtropical regions of the world. Dengue cases in the Americas have increased over time from 1980 to 2007, with Mexico being the third country with the highest number of dengue cases (San Martín et al., 2010). *Aedes aegypti* is widely distributed in Mexico including the urban communities in the southern, western and eastern regions, extending along the Gulf Coast as far north as Matamoros, Mexico, and to the southern half of the United States (de Lourdes Muñoz et al., 2013; Hahn et al., 2017).

Along the U.S. border, DENV is endemic only in the southern region of Texas (Rio Grande Valley), even though *Ae. aegypti* is found in almost all urban U.S. border communities. Dengue sporadic outbreaks in the Rio Grande Valley in the last 20 years have been coincident with an explosive epidemic of dengue in the Mexican border communities. For instance, dengue outbreak in Brownsville, Texas reported less than 100 cases (2005 and 2013 together) and was inferior to the over 7000 and 5500 dengue cases, including dengue severe disease, reported in 2005 and 2013, respectively in the Mexican State of Tamaulipas, which borders Rio Grande Valley ((Ramos et al., 2008; Thomas et al., 2016). Only a community located west of the Rio Grande Valley in Yuma, Arizona have reported dengue and it was determined that all the cases (n=93) were imported from the Mexican border community of Sonora (Jones et al., 2016).

The possible imported or endemic dengue outbreaks in other U.S. border cities such as El Paso from dengue epidemics in the Mexican border is latent. More recently, data accumulated suggested that DENV is endemic in Ciudad Juarez, Mexico, an urban community in the state of Chihuahua located in Northwestern Mexico and is the second-largest community located along the U.S - Mexico border. The first reported evidence was the detection of DENV ribonucleic

acid (RNA) in *Ae. aegypti* mosquitoes in Ciudad Juarez (De la Mora et al. 2010*)*. Subsequently, serological evidence of DENV infection in humans was also reported in the Anapra neighborhood of Ciudad Juarez, including seroconversions to DENV infection in 8.33% (n=4) of 48 individuals who were negative for DENV antibody during the baseline survey (Palermo et al., 2019). Furthermore, several dengue cases have been reported in Ciudad Juarez more recently (DGE, 2016, 2017, 2019). However, there is limited evidence if DENV was isolated from viremic patients in Ciudad Juarez. This step is critical to determine if *Ae. aegypti* populations not only from the dengue outbreak area, but also from neighbor communities along the U.S-Mexican border are competent to transmit the virus. This information can provide an understanding of the dynamics and risk of dengue transmission in an area and contribute to the development of more efficient mosquito control activities at local or state level. Based on this information, the chapter was divided into two aims:

Aim 1: Characterization of a dengue virus serotype 1 isolated from a patient in Ciudad Juarez, Mexico

Aim 2: Vector competence of *Ae. aegypti* from El Paso and Rio Grande to the Mexican DENV-1

4.2. Characterization of a Dengue virus serotype 1 isolated from a patient in Ciudad Juarez, Mexico

4.2.1. Material and methods

4.2.1.1 Patient and sample collection and processing

A 64-year-old female housewife who resided in Felipe Angeles, Ciudad Juarez, Chihuahua (Mexico) experienced a febrile illness in November 2015 that was characterized by an acute onset of chills, severe headache, fever, diarrhea, joint pain, bad taste in the mouth, and decreased appetite. She sought medical attention and medication was administered for symptomatic treatment. A venous blood sample was collected during the acute phase of illness from the patient with a 6 mL vacutainer tube containing an Ethylenediamine tetraacetic acid (EDTA) from the arm of the patient at her residence in the neighborhood of Felipe Angeles, Ciudad Juarez, Chihuahua, Mexico (**Figure 4.1**). The blood sample was centrifuged at 3000 gs for 10 minutes and the plasma was stored in aliquots of 0.5 mL at -80ºC until tested for arboviruses in C6/36 and Vero-76 cells. A convalescence blood sample was not available for antibody testing. In addition, the female patient did not report any travel history prior to the onset of symptoms. The de-identified medical history and deidentified blood sample was provided by one of the authors (AMC) to another author (DMW) at the University of Texas at El Paso (UTEP), Texas to test for arboviruses. The patient was enrolled in accordance to the Ethic Committee at the Institute of Biomedical Sciences of the Autonomous University of Juarez, Mexico using a written informed consent form and a questionnaire to obtain demographic and clinical information.

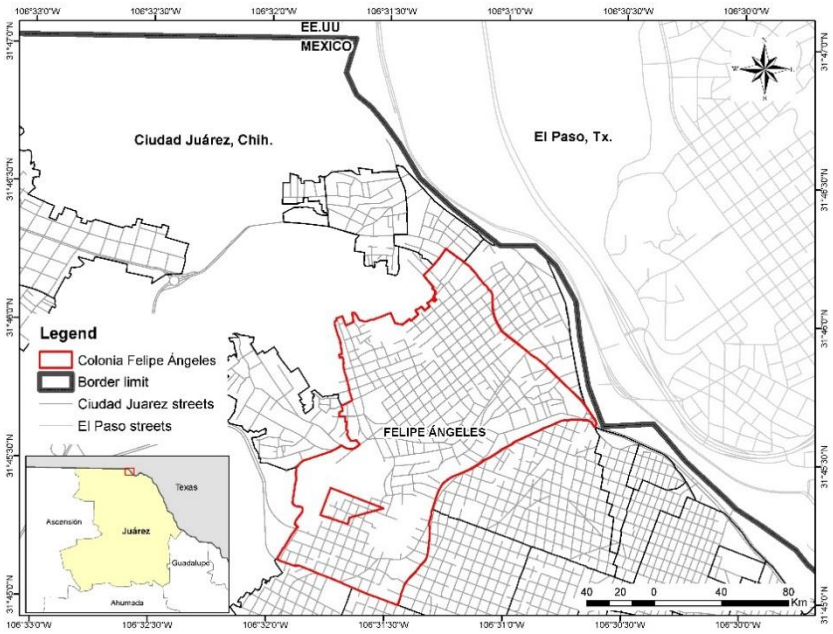


Figure 4.1. Map of Ciudad Juarez, Mexico highlighting the adjoining neighborhood of Felipe Angeles, Ciudad Juarez, Chihuahua 32100**.**

4.2.1.2 Viral isolation

The patient's plasma sample was diluted 1:10 in cell culture maintenance medium (Minimum Essential Medium (MEM) supplemented with 2% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% non-essential amino acids). Two hundred microliters of the diluted plasma sample were inoculated onto confluent monolayers of Vero-76 and C6/36 cells propagated in a T-25 cm^2 flask and incubated for 1 hour at 37ºC and 28ºC in the presence of 5% CO_2, respectively. Then, 5 mL of the maintenance medium was added to each culture and the cells and inoculum were incubated for 7 days at 37ºC and 28ºC at 5% CO_2. Confluent monolayers of Vero-76 and C6/36 cells were inoculated with maintenance medium to serve as controls. The cells were observed for evidence of viral cytopathic effect (CPE) once daily through an inverted microscope. After 7 days post-inoculation, the Vero and C6/36 cells were scraped off the flasks and the suspensions were clarified by centrifugation at 3000 gs for 10

minutes at 4ºC. The clarified cells and supernatants were stored at -80°C and labeled as passage 1 (p-1). The cell pellets were resuspended in phosphate buffer saline (PBS) 1X and aliquots of 20 µL were spotted onto the ringed areas of slides and dried and fixed in cold acetone at -20°C for 10 minutes and stored at -20°C for subsequent testing of the cell pellets for selected arboviruses using an immunofluorescence assay.

4.2.1.3 Immunofluorescence assay (IFA)

The IFA was performed on the cell pellet fixed to the ringed area of the slides using polyclonal antibodies for West Nile, St Louis encephalitis, DENV serotype 1 and 2 (DENV-1, DENV-2), Chikungunya, Western equine encephalitis, and La Crosse. Also, DENV-1 monoclonal antibody (15F3) ascites was included in the test. Briefly, 10 µL of polyclonal or monoclonal antibodies were added to the cell pellets of the slide and incubated at 37ºC for 1 hour. Then, the slides were washed twice in PBS 1X, dried and then 10 µL of goat anti-mouse IgG antibody conjugated with Fluorescein Isothiocyanate (1:100 diluted in PBS 1X) was added and incubated at 37ºC for 1 hour. DAPI (4', 6-diamidino-2-phenylindole) was used as a counterstain (1:1000 dilution). The slides were washed twice in PBS 1X, dried, and a drop of a glycerol solution (9 parts glycerol + 1-part PBS) was added onto the ringed area of the slides. Slides were examined for fluorescence using a 20x objective on a fluorescent microscope (Nikon, Ti-S). Slide controls containing known infected cells with each of the test viruses and/or uninfected cells were subjected to the same procedure. A *Flavivirus* was detected by IFA using *Flavivirus* polyclonal antibodies and shown to be DENV- 1 by using DENV-1 monoclonal antibody.

4.2.1.4 Viremia levels

Tenfold dilutions of the patient's plasma sample were prepared in maintenance media, and 50 µL of each dilution was inoculated into a suspension of baby hamster kidney cells (BHK-21 clone 15) propagated in 24-well plates. A plaque assay was then performed to determine the titer of the DENV-1 isolate as described previously (Morens et al., 1985).

4.2.1.5 Molecular identification

RNA was extracted from the Vero-76 and C6/36 cell infected with DENV-1 (p1) using the QIAamp viral RNA kit (Qiagen, Valencia, CA) following the manufacturer's instructions. A generic reverse transcription-polymerase chain reaction (RT-PCR) assay was performed to detect the nucleic acid for *Flaviviruses*, as previously described (Kuno et al., 1998). The amplicons obtained were purified and sequenced using the BigDye® Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), with the sequencer 3730xl DNA analyzer (Applied Biosystems). Sequences were edited with the Sequencher 4.6 software (Gene Codes Corporation, Ann Arbor, MI), and compared to other viral sequences deposited in the GenBank database using the BLASTn software.

4.2.1.6 Envelope gene sequencing

The pre-Membrane (preM) and envelope (E) genes of the DENV-1 isolate (p1) were amplified by RT-PCR (Díaz et al., 2006; Warrilow et al., 2012) . Phylogenetic analysis of the E gene of DENV-1 was performed using the neighbor-joining and the Maximum probability analysis implemented in MEGA7 (Kumar et al., 2016). The best fit model (GTR+ I + G) was determined by the Mr. Model test software. Clades were evaluated by bootstrap analyses with 1000 replicates for maximum probability analyses. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise

distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with the superior log-likelihood value.

4.2.1.7 Whole Viral-Genome Sequencing

DENV-1 RNA was prepared using the NEBNext Ultra II RNA Library Prep Kit (New England BioLabs, Ipswich, MA). Finished libraries were quality checked on an Agilent Bioanalyzer (Agilent, Santa Clara, CA) and quantified by real-time PCR. Libraries were pooled and sequenced on a NextSeq 550 (Illumina, San Diego, CA) using the High-output kit (Illumina, San Diego, CA) and a paired-end 75 base read protocol. Raw reads were filtered to remove low quality reads and adapter sequences using the Trimmomatic software program (Bolger et al., 2014). The read assembly program ABySS (2) was used to assemble the filtered reads into longer de novo contigs to assemble the genome.

Sixty-eight DENV-1 sequences, as well as DENV-2 New Guinea C to root the tree, were downloaded from GenBank (**Appendix 2**). The resulting set of 69 strains from GenBank, plus the 2015 isolate from Ciudad Juarez, Mexico were aligned using Clustal Omega in MegAlign Pro version 17.0.0 (DNASTAR Inc, Madison, WI) and trimmed to remove the 5’ and 3’ UTRs. Maximum likelihood analysis was performed using the phangorn package (Schliep, 2011) in R version 3.5.3 (R Core Team 2017). The GTR+G+I model of nucleotide substitution was selected from the 24 options analyzed as part of the ModelTest analysis in phangorn because of minimizing the AIC score. A maximum likelihood tree was generated using a rooted UPGMA tree as a starting point, and bootstrapping was performed with 1,000 iterations. The resulting tree was visualized using iTOL version 5.7 (Letunic & Bork, 2019).

4.2.1.8 Replication curve

The infectivity replication curve of the DENV-1 isolate was determined in Vero-76 and C6/36 cells by plaque assay in BHK-21 clone 15 cells (Morens et al., 1985) . The C6/36 cells were grown in a T-75 cm^2 flask and inoculated with the DENV-1 isolate (p-1 from C6/36 cells supernatant) at a multiplicity of infection (MOI) 0.01. Cells were harvested once CPE became evident at 7 days post-infection (dpi), and aliquots of the clarified supernatant (p-2) were stored at -80ºC until used to determine the virus infectivity replication curves. Briefly, Vero-76 and C6/36 cells were inoculated in triplicate with the DENV-1 suspension (p-2) at MOI 0.01 and incubated at 37ºC and 28ºC, respectively for 1 hour. Then, the inoculum was removed, and the cells were washed three times with sterile PBS 1X pH 7.4. Five milliliters of maintenance media were added to cells of each flask and a 0.5 mL aliquot was collected immediately (time point 0) and replaced with 0.5 mL of new media. Cells were incubated as mentioned above and aliquots were collected and replaced with new media every day until 10 dpi. Timepoint aliquots were tested to determine virus infectivity titers by plaque assays on BHK-21 clone 15 cells. Statistical differences in the viral replication titers were determined by using the Student's T-Test.

4.2.2. Results

A *Flavivirus* was isolated from the patient's plasma sample in the C6/36 cells based on the detection of viral antigen by IFA using polyclonal *Flavivirus* antibodies (**Figure 4.2**). The *Flavivirus* isolate was further identified as DENV-1 using DENV-1 monoclonal antibody (**Figure 4.2**) and by sequencing of the generic *Flavivirus* RT-PCR amplicon (data not shown). The infectivity titer of the DENV-1 in the plasma of the patient was 2.69 log $_{10}$ PFU/mL at the time the blood sample was drawn.

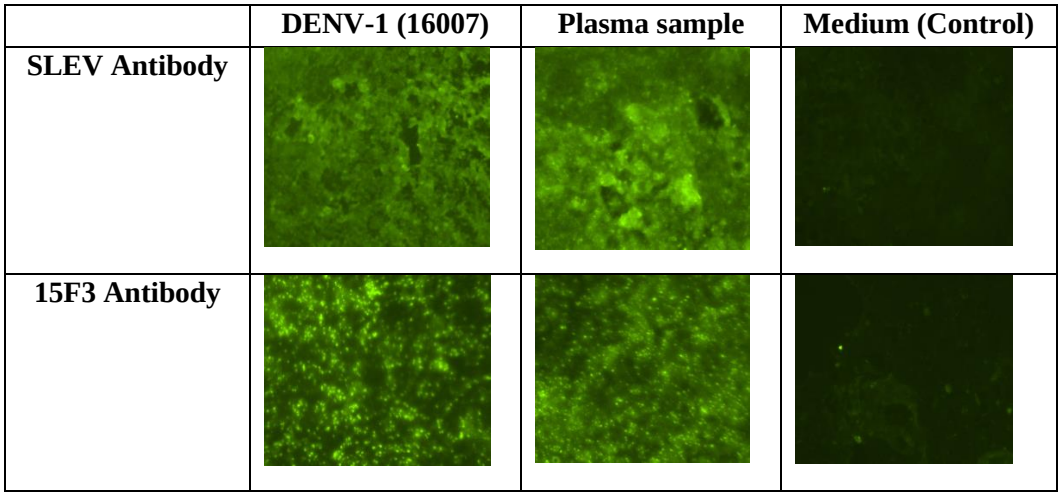


Figure 4.2. Indirect immunofluorescent antibody assay in C6/36 cells (200X magnification). Cells were inoculated with DENV-1**,** human plasma, and cell culture medium. Viral antigen was detected by using polyclonal antibodies to St Louis encephalitis virus (top row) and monoclonal antibody (15F3) to DENV-1 (bottom row).

In vitro viral replication of the DENV-1 (p-2) was compared in Vero and C6/36 cells up to 10 dpi. DENV-1 (p-2) replication in C6/36 cells was approximately 3 logs higher in C6/36 cells than in Vero-76 cells from 2 to 10 dpi ($p<0.05$), reaching the highest titer (2 x10^6 PFU/mL), after 7 dpi (**Figure 4.3**).

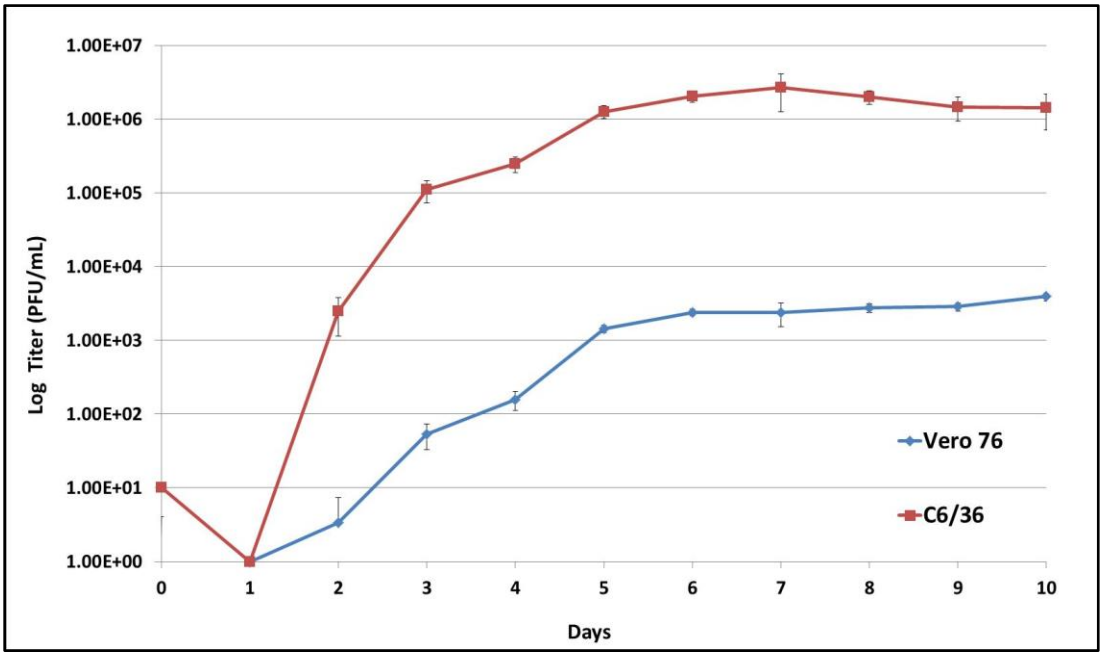


Figure 4.3. Replication curves of the Ciudad Juarez DENV-1 isolate in Vero-76 and C6/36 cells.

Phylogenetic analysis of the E gene indicated that the DENV-1 isolate clustered in the Genotype V and grouped with other Central American strains of DENV-1 (**Figure 4.4**) and with other DENV-1 strains isolated in Mexico (Tamaulipas, Yucatan, and Morelos) and Texas (Brownsville). Furthermore, a full genome analysis showed that the DENV-1 isolate clustered with Central American DENV-1 strains (**Figure 4.5**).

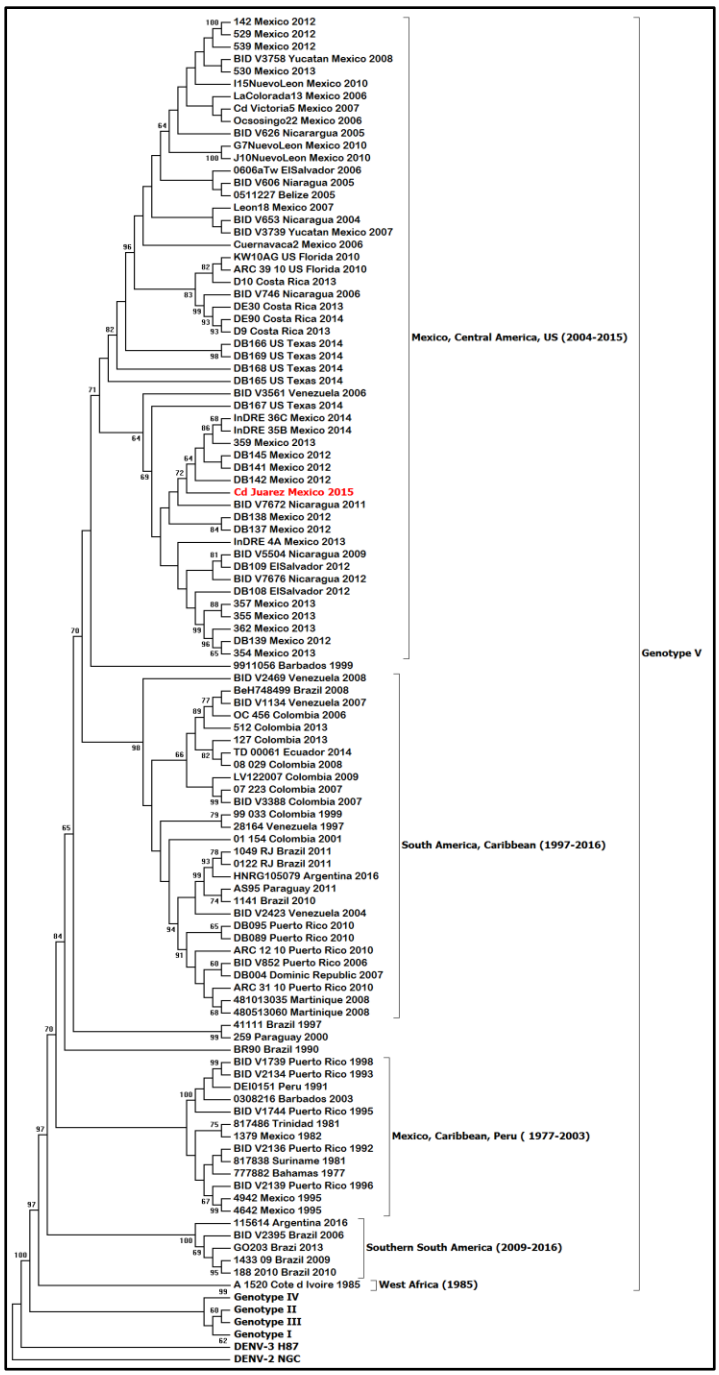


Figure 4.4. Molecular phylogenetic analysis by Maximum Likelihood method derived from 100 DENV-1 envelope glycoprotein gene sequences. DENV-1 Genotypes (I-V) and isolates are shown by using brackets. The tree was rooted with prototype strains of DENV-3 (H87) and DENV-2 NGC). DENV-1, strain Ciudad Juarez appears red highlighted. The tree with the highest log likelihood (-12372.1590) is shown.

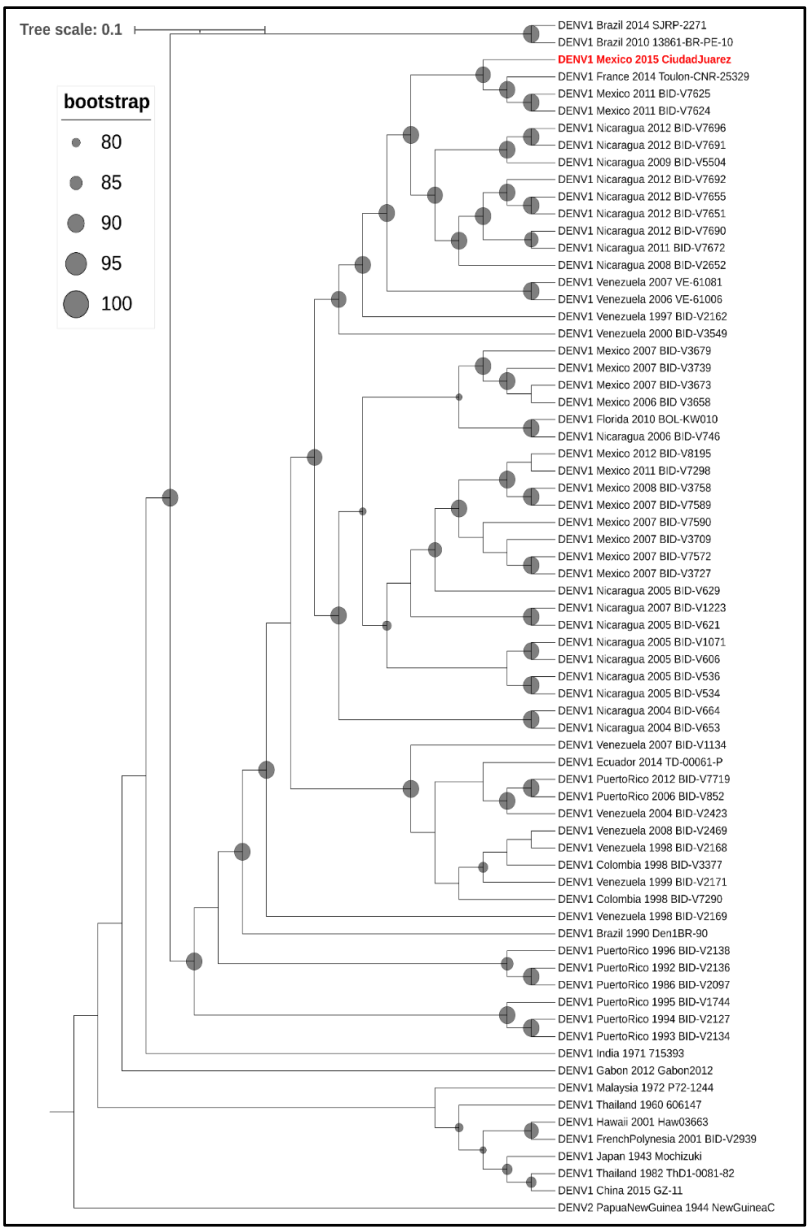


Figure 4.5. Phylogenetic analysis of DENV-1. Maximum-likelihood tree based on the full genome sequences (minus the 5' and 3' UTRs) of 68 strains of DENV-1 from GenBank, along with the 2015 Ciudad Juarez isolate (highlighted with red and bold text). DENV-2 strain New Guinea included to root the tree. In the cladogram, branch lengths are ignored but bootstrapping values ≥80 are represented as proportionately sized grey circles at the relevant nodes.

Amino acid diversity of the DENV-1 isolate was compared with other Mexican DENV-1 strains (**Table 4.1**). The DENV-1 isolate was unique amongst the other Mexican strains in the amino acid alignment due to five amino acid changes in the NS1 (D139E), NS2 (L41F, K218R), and NS3(V332A, R338K) proteins (**Table 4.1**).

Table 4.1. Aminoacid changes in the Mexican DENV-1 strains in comparison to the DENV-1 Ciudad Juarez strain.

Tree Name	Collection Year	Strain Name	GenBank Accession No.	NS1	NS2A		NS3	
				139	41	218	332	338
DENV1_Mexico_2006_BID_V3658	2006	BID_V3658	GU131958.1	D	L	K	V	R
DENV1_Mexico_2007_BID-V3673	2007	BID-V3673	GU131964.1	D	L	K	V	R
DENV1_Mexico_2007_BID-V3679	2007	BID-V3679	GU131966.1	D	L	K	V	R
DENV1_Mexico_2007_BID-V3709	2007	BID-V3709	GQ868513.1	D	L	K	V	R
DENV1_Mexico_2007_BID-V3727	2007	BID-V3727	GQ868521.1	D	L	K	V	R
DENV1_Mexico_2007_BID-V3739	2007	BID-V3739	GQ868527.1	D	L	K	V	R
DENV1_Mexico_2007_BID-V7572	2007	BID-V7572	KJ189317.1	D	L	K	V	R
DENV1_Mexico_2007_BID-V7589	2007	BID-V7589	KJ189323.1	D	L	K	V	R
DENV1_Mexico_2007_BID-V7590	2007	BID-V7590	KJ189324.1	D	L	K	V	R
DENV1_Mexico_2008_BID-V3758	2008	BID-V3758	GQ868537.1	D	L	K	V	R
DENV1_Mexico_2011_BID-V7298	2011	BID-V7298	KJ189306.1	D	L	K	V	R
DENV1_Mexico_2011_BID-V7624	2011	BID-V7624	KJ189348.1	D	L	K	V	R
DENV1_Mexico_2011_BID-V7625	2011	BID-V7625	KJ189349.1	D	L	K	V	R
DENV1_Mexico_2012_BID-V8195	2012	BID-V8195	KJ189368.1	D	L	K	V	R
DENV1_Mexico_2015_CiudadJuarez	**2015**	**Ciudad Juarez**	**This study**	**E**	**F**	**R**	**A**	**K**

4.3. Vector competence of Ae. aegypti from El Paso and Rio Grande to the Mexican DENV-1 isolate

4.3.1. Material and Methods

4.3.1.1. Standard rearing techniques of *Ae. aegypti*

Ae. aegypti mosquitos from El Paso and Rio Grande were reared at the UTEP Insectary (Biology Building, Room 207) as described previously (Chapter III, Section 3.2.1.1).

4.3.1.2. Oral mosquito infections

F3 generations were tested for DENV-1 oral infection as mentioned previously (Chapter III, Section 3.2.1.4). Two different viral doses (4 and 6 $\log_{10}$ PFU/mL) were used to evaluate possible differences of the viral dose in the susceptibility of *Ae. aegypti* strains. Briefly, 6 $\log_{10}$ PFU/mL of freshly propagated DENV-1 was diluted 2 logs in maintenance media (4 $\log_{10}$ PFU/mL) without FBS. Each viral dose (4 or 6 $\log_{10}$ PFU/mL) was mixed with of 2 mL of defibrinated sheep blood (Colorado Serum Company, Denver, CO). An aliquot of each infectious blood meal was collected before and after feeding the mosquitoes to determine the amount of DENV-1 in the virus-blood mixtures.

4.3.1.3. Mosquito processing

Each mosquito was processed as follows: abdomen and thorax were tested to determine infectivity rates; legs and wings for disseminated infectivity rates; and saliva for transmission rates (Chapter III, Section 3.3.1.3)

4.3.1.4. Viral detection and quantification

Mosquito bodies, legs/wings, and saliva were tested by plaque assays, viral isolation and IFAT as stated previously (Chapter III, Section 3.2.1.6).

4.3.1.5. Statistical Analysis

Infectivity rates were calculated as the proportion of total number of mosquito bodies tested with the number of positive-virus bodies. Dissemination rates were calculated as the rate of mosquitoes with infected bodies that also have infected legs. Transmissions rates were defined as the rate of infected bodies of mosquitoes that also have virus positive saliva. Analysis of variance (ANOVA) was used to determine significant differences between mosquito strains, DENV-1 viral dose, infectivity and dissemination rates, and extrinsic incubation periods.

4.3.2. Results

4.3.2.1. *Ae. aegypti* from the U.S. border are susceptible to the Mexican DENV-1

Ae. aegypti mosquitoes from the two U.S. border regions (El Paso and Rio Grande) were susceptible to DENV-1 Mexican isolate infection after ingesting 6 log $_{10}$ PFU/mL (**Figure 4.6**). El Paso *Ae. aegypti* mosquitoes had an infectivity rate of 93.33% (14/15), 87.5% (14/16), and 87.5% (14/16) at 7, 14 to 21dpi respectively. In case of the mosquitoes from Rio Grande, an infectivity rate of 76.92% (10/13), 92.31% (12/13), and 84.61% (11/13) was observed at 7, 14, and 21 dpi respectively. Overall, no significant differences ($p>0.05$) were found between the two *Ae aegypti* strains and the Mexican DENV-1 infectivity rate at 7, 14, and 21 dpi. Finally, DENV-1 infectivity rates of each *Ae. aegypti* strain did not differ significatively through the incubation time.

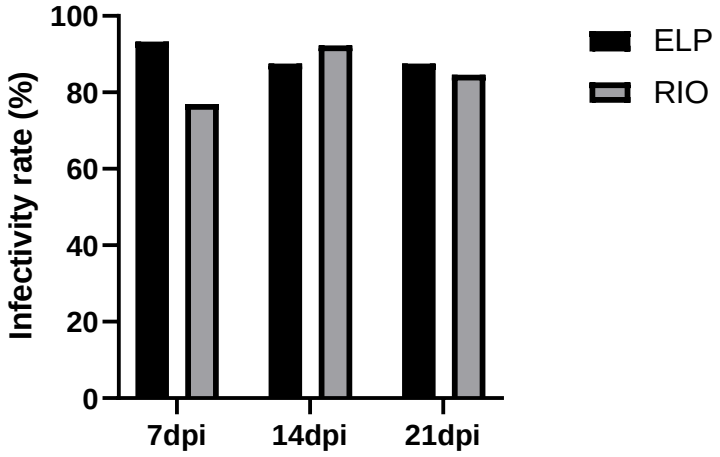


Figure 4.6. Infectivity rate of El Paso and Rio Grande *Ae. Aegypti* after ingesting 6 log $_{10}$ PFU/mL of DENV-1 at 7, 14, 21dpi.

In addition, both *Ae. aegypti* strains were exposed to a lower viral dose (4 log 10 PFU/mL) to evaluate if the DENV-1 susceptibility in *Ae. aegypti* might experience some variation. El Paso *Ae aegypti* had an infectivity rate of 54.55% (12/22), 54.16% (13/24), and

63.63% (14/22) at 7, 14 and 21dpi, respectively, while Rio Grande *Ae. aegypti* had an infectivity rate of 54.54% (6/11), 16.6% (2/12), and 50% (6/12), respectively (**Figure 4.7**). The two *Ae. aegypti* strains didn't differ in the infectivity rate at 7 and 21dpi, but significant differences between the two *Ae. aegypti* strains and their infectivity rates were found at 14dpi only ($p.<0.05$).

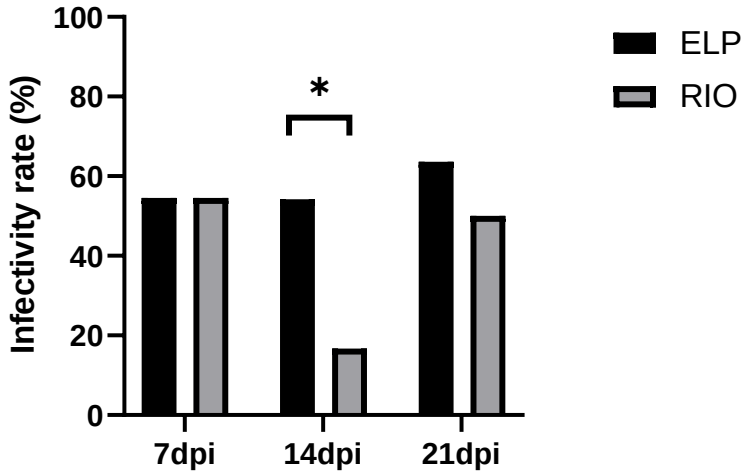


Figure 4.7. Infectivity rate of El Paso and Rio Grande *Ae. aegypti* after ingesting 4 log $_{10}$ PFU/ mL of DENV-1 at 7, 14, 21dpi.

4.3.2.2. DENV-1 dose significant affect the infectivity rates in the *Ae. aegypti* from the U.S. border

The overall infectivity rate after ingesting 6 and 4 log $_{10}$ PFU/mL was 89.36% (42/47) and 57.35% (39/68), respectively for El Paso *Ae. aegypti* mosquitoes, and of 84.62% (33/39) and 40% (14/35), respectively for the Rio Grande mosquitoes (**Figure 4.8**). Viral ingestion dose had a significant effect on the overall infectivity rate of both *Ae. Aegypti* strains. A lower DENV-1 ingested dose (4 $\log_{10}$ PFU/mL) caused a significant decrease in the infectivity rate of the *Ae aegypti* mosquitoes from El Paso, from 89.36 % to 57.35% ($p=0.0002$). A similar decrease was

also found in the Rio Grande *Ae aegypti* infectivity rate, ranging from 84.62% to 40% ($p<0.0001$).

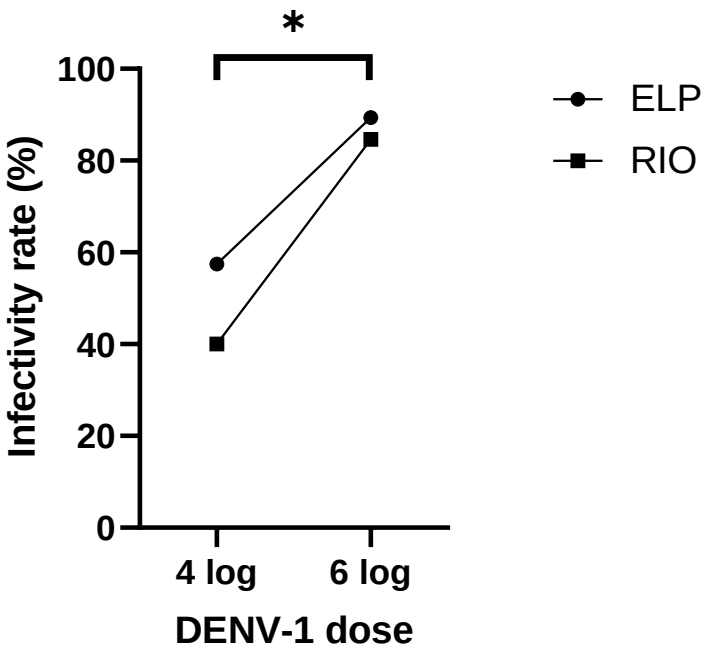


Figure 4.8. Overall infectivity rate of El Paso and Rio Grande *Ae. aegypti* after ingesting 6 log $_{10}$ PFU/ mL or 4 log $_{10}$ PFU/ mL of DENV-1

4.3.2.3. Mexican DENV-1 isolate can disseminate in the *Ae. aegypti* from the U.S. border

Disseminated infection rates of DENV-1 in *Ae. aegypti* mosquitoes from El Paso and Rio Grande after ingesting a 6 log $_{10}$ PFU/mL viral dose were 78.57% (11/14) and 80% (8/10), respectively at 7 dpi (**Figure 4.9**). Disseminated infection rates reached 100% (14/14) at 14dpi and were maintained until 21dpi for both mosquito strains. A significant increase $p<0.05$) in the disseminated infection rate was observed from 7 to 14 dpi for both *Ae. aegypti* strains. Finally, no significant differences ($p>0.05$) in the disseminated infection rates were found between both mosquito strains at 7, 14, and 21 dpi.

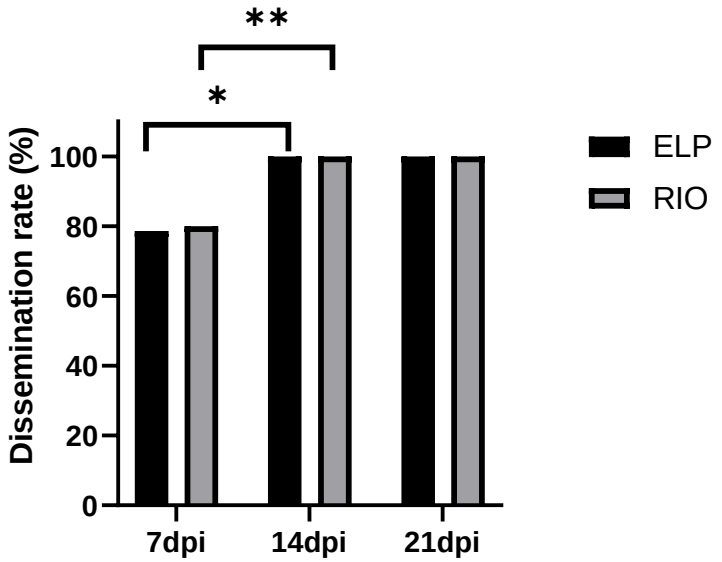


Figure 4.9. Disseminated infection rates of El Paso and Rio Grande *Ae. aegypti* after ingesting 6 log $_{10}$ PFU/ mL of DENV-1 at 7, 14, 21dpi.

A viral dose ingested by the mosquitoes of 4 $\log_{10}$ PFU/mL caused a disseminated infection rate of 66.67% (8/12) and 16.67% (1/6) at 7 dpi in mosquitoes from El Paso and Rio Grande respectively (**Figure 4.10**). Even though differences were observed in the disseminated infection rates in both strains at 7 dpi, the difference was not significant, due to the low number of individuals. Furthermore, disseminated infection rates increased to 100% (13/13) at 14dpi for both *Ae. aegypti* strains in comparison to the rates found at 7 dpi ($p<0.05$). Finally, the disseminated infection rates were not associated to the mosquito strains during the period of incubation ($p>0.05$) after ingesting 4 log $_{10}$ PFU/mL of viral dose.

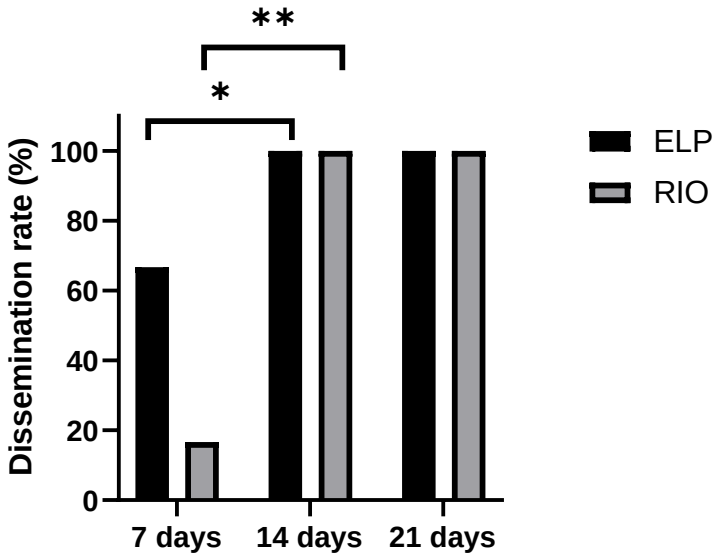


Figure 4.10. Disseminated infection rate of El Paso and Rio Grande *Ae. aegypti* after ingesting 4 log $_{10}$ PFU/ mL of DENV-1 at 7, 14, 21dpi.

Overall, the DENV-1 disseminated infection rates reached 100% for both mosquito strains at 14dpi using the two DENV-1 viral doses (4 and 6 log $_{10}$ PFU/mL). Early days after infection (7 dpi), disseminated infection rates were mainly over 50% for El Paso and Rio Grande with the two viral doses, with the only exception of Rio Grande strain (16.67%) after ingesting 4 log$_{10}$ PFU/mL viral dose. A significant increase $p<0.05$) in the disseminated infection rate was observed from 7 to 14 dpi for both *Ae. aegypti* mosquito strains and viral doses (**Figure 4.9** and **4.10**).

4.3.2.4. *Ae. aegypti* mosquitoes from the U.S. Border have low rates of DENV-1 transmission

DENV-1 was recovered in the saliva of a few *Ae. aegypti* mosquitoes from El Paso and Rio Grande. Overall transmission rates of both *Ae. aegypti* strains was low and reported as

5.71% (2/35) and 3.23% (1/31) in mosquitoes from El Paso (after ingesting 4 $\log_{10}$ PFU/mL viral dose), and Rio Grande (6 $\log_{10}$ PFU/mL viral dose), respectively. (**Figure 4.11B and 4.11C**).

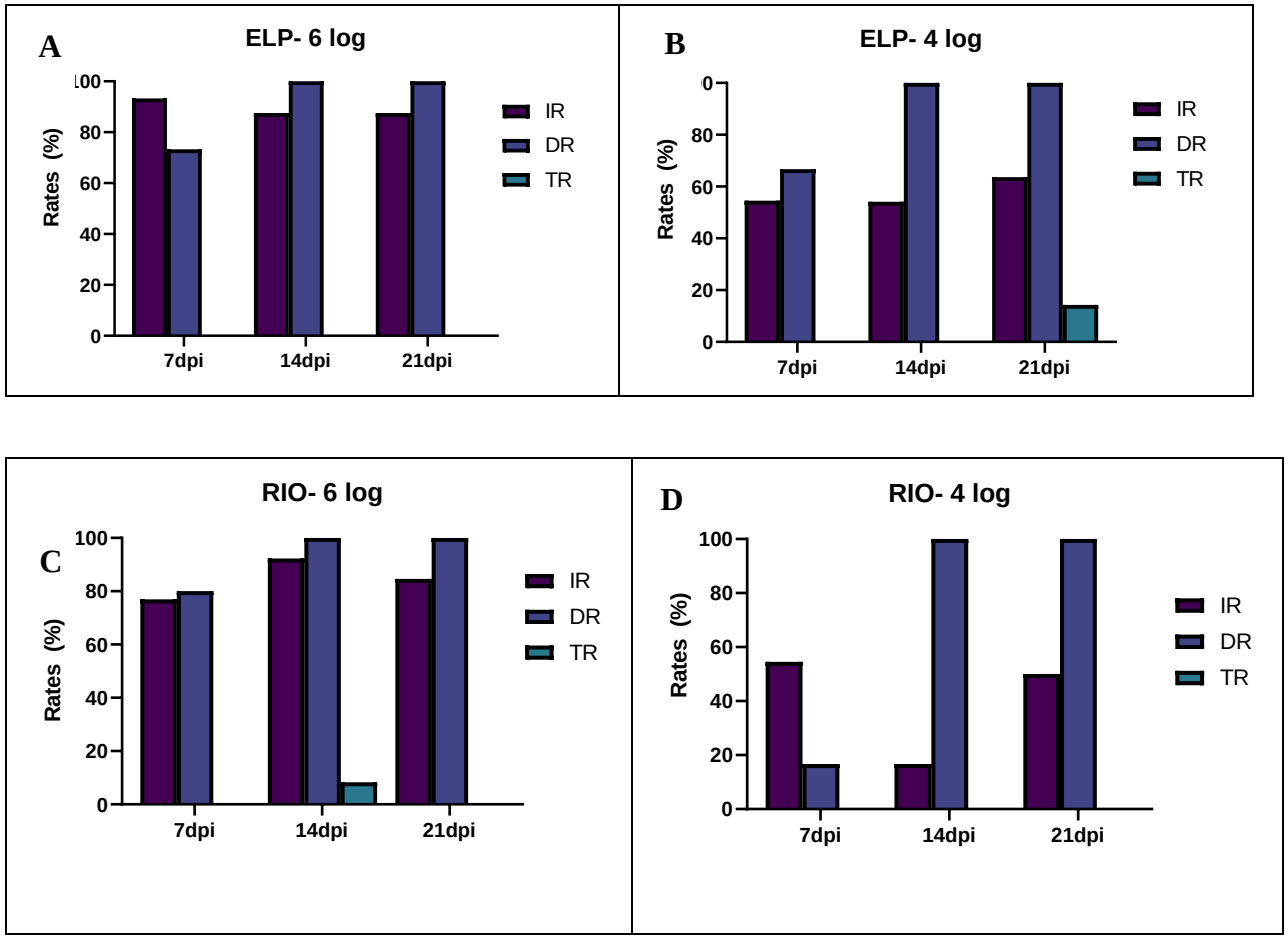


Figure 4.11. Infection (IR), dissemination (DR), and transmission rates (TR) of DENV-1 by *Ae. aegypti* from the U.S. border region at 7, 14, 21dpi. El Paso (**A, B**) and Rio Grande (**C, D**) *Ae. aegypti* ingested 6 log $_{10}$ PFU/mL of DENV-1 (**A, C**) or 4 log $_{10}$ PFU/ mL (**B, D**).

4.3.2.5. DENV-1 replication dynamics in *Ae aegypti* bodies from the U.S. border

Infectivity titers in *Ae. aegypti* bodies were determined by plaque assays in BHK-15 cells, after mosquitoes ingested two viral doses (4 and 6 log $_{10}$ PFU/mL). Infected *Ae. aegypti* mosquitoes with 6 log $_{10}$ PFU/mL of DENV-1 had a mean body viral titer of 4.198 log $_{10}$ PFU/mL at 7 dpi. Then titer slightly increased to 4.50 and 4.66 $\log_{10}$ PFU/mL at 14 and 21 dpi,

respectively (**Figure 4.12A**). Viral titers/mosquito had more variation at 7 dpi (2.6-5.04 log_{10} PFU/mL) than at 14 dpi (3.6-5.11 log_{10} PFU/mL) and 21 dpi (4.38-4.94 log_{10} PFU/mL). Mean viral titers in mosquito bodies through the extrinsic incubation period were not significant ($p>0.05$). A similar trend in viral titer in Rio Grande *Ae. aegypti* mosquito bodies was found after ingesting 6 log_{10} PFU/mL of DENV-1(**Figure 4.12B**). Briefly, mosquito bodies had a mean viral titer of 4.168, 4.339, and 4.482 log_{10} PFU/mL at 7, 14, and 21 dpi, respectively. No association was observed between mean viral titer in mosquito bodies from Rio Grande and days post infection ($p>0.05$).

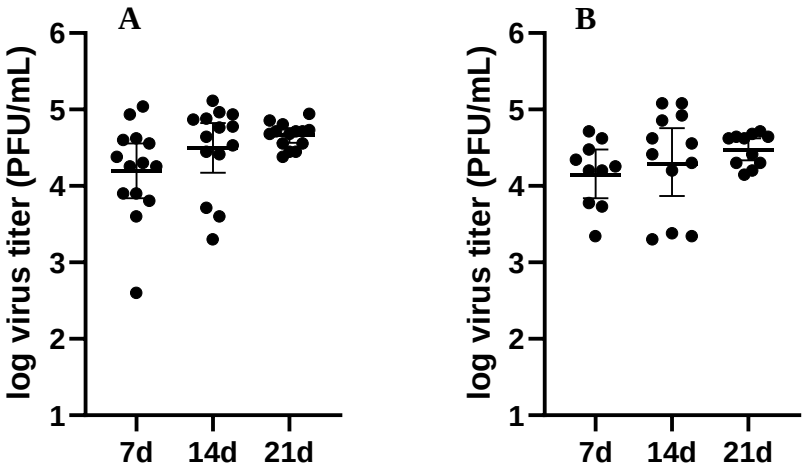


Figure 4.12. Virus titer in bodies of infected *Ae. aegypti* from El Paso (**A**) and Rio Grande (**B**) after ingesting 6 log_{10} PFU/ mL of DENV-1

DENV mean titers in *Ae. aegypti* mosquito bodies from El Paso and Rio Grande after ingesting 4 log_{10} PFU/mL of DENV-1 showed a slightly increased titer through the period of incubation (**Figure 4.13**). However, again the increase in the mean viral titer was not significant in El Paso mosquitoes ($p>0.05$) through time (3.42, 3.758, and 3.819 log_{10} PFU/mL at 7, 14, and 21 dpi respectively). In case of Rio Grande *Ae. aegypti* mosquitoes, an increase in the mean viral

titer was observed at 21 dpi (4.528 log $_{10}$ PFU/mL) in comparison to 7 and 14dpi (3.253 and 3.228 log $_{10}$ PFU/mL). However, the low number of infected mosquitoes at 7 and 14dpi affected the statistical analysis.

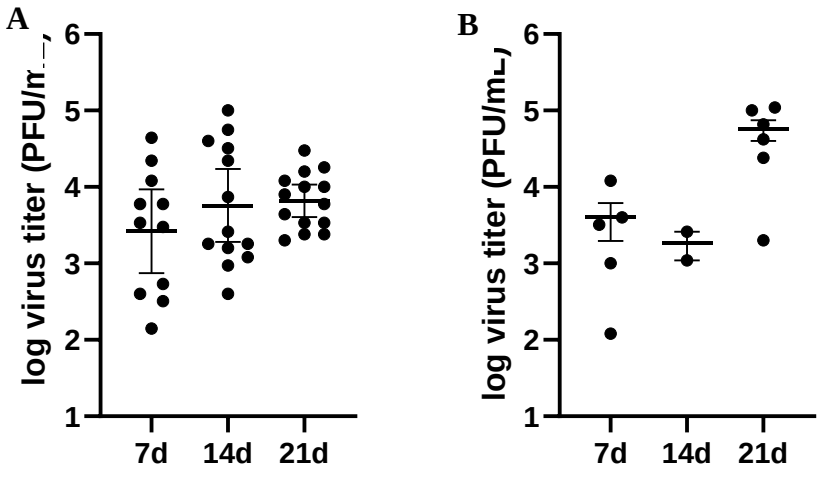


Figure 4.13. Virus titer in bodies of infected *Ae. aegypti* from El Paso (**A**) and Rio Grande (**B**) after ingesting 4 log $_{10}$ PFU/ mL of DENV-1.

4.3.2.6. DENV-1 dose affects the viral titers in *Ae. aegypti* mosquitos' bodies

Viral titer in *Ae. aegypti* bodies during the incubation period was significantly affected by the ingested viral dose at 7, 14 and 21 dpi (**Table 4.2**). El Paso mosquitoes had a significant decrease ($p<0.05$) in the mean viral titer at each time point (0.7-.0.8 $\log_{10}$) after ingesting 4 $\log_{10}$ PFU/mL in comparison to the 6 $\log_{10}$ PFU/mL viral dose ($p<0.05$). *Ae. aegypti* mosquitoes from Rio Grande also had a decrease in viral titers at 7 dpi ($p<0.05$) and 14 dpi after ingesting 4 log $_{10}$ PFU/mL viral dose in comparison to 6 $\log_{10}$ PFU/mL viral dose. However, the mean viral titers (4.482 $\log_{10}$ PFU/mL vs 4.528 $\log_{10}$ PFU/mL) were not different at a long incubation period (21dpi) of after ingesting 6 and 4 $\log_{10}$ PFU/mL viral dose.

Table 4.2. DENV-1 means titer in *Ae. aegypti* mosquitoes from El Paso and Rio Grande at 7, 14, and 21 dpi after ingesting a viral dose of 4 log $_{10}$ PFU/mL or 6 log $_{10}$ PFU/mL.

		6 log $_{10}$ PFU/mL	4 log $_{10}$ PFU/mL	
ELP	7dpi	4.198	3.42	*p<0.05
	14dpi	4.497	3.758	*p<0.05
	21dpi	4.66	3.819	*p<0.05
RIO	7dpi	4.168	3.253	*p<0.05
	14dpi	4.339	3.228	*p<0.05
	21dpi	4.482	4.528	p>0.05

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4.3.2.7. Infected DENV-1 *Ae. aegypti* mosquitoes from the U.S. border did not differ in the body viral titers

Finally, no significant difference (p>0.05) was observed between the mean viral titers of El Paso and Rio Grande mosquito bodies at each time post infection (7, 14, and 21 dpi) after ingesting 6 $\log_{10}$ PFU/mL viral dose (**Table 4.3**). Also, no significant difference was observed in the mean viral titer for both mosquito strains through the incubation time after ingesting 4-$\log_{10}$ viral dose of DENV-1. These results indicated that the mean body titers were not affected by the two *Ae. aegypti* strains after being orally infected with 4 or 6 $\log_{10}$ PFU/mL of the DENV-1.

Table 4.3. DENV-1 mean body titers of *Ae. aegypti* strains (ELP vs RIO) after ingesting 4 $\log_{10}$ PFU/mL or 6 $\log_{10}$ PFU/mL.

		ELP	**RIO**	
6 log10	7dpi	4.198	4.168	p>0.05
PFU/mL	14dpi	4.497	4.339	p>0.05
	21dpi	4.66	4.482	p>0.05
4 log10	7dpi	3.42	3.253	p>0.05
PFU/mL	14dpi	3.758	3.228	p>0.05
	21dpi	3.819	4.528	p>0.05

4.3.2.8. Viral replication dynamics in *Ae. aegypti* legs

Viral titer in mosquito legs from El Paso increased through time after ingesting a viral dose of 6 $\log_{10}$ PFU/mL of DENV-1 (**Figure 4.14**). Briefly, mean viral titer in legs were 2.383, 2,651, and 2.869 $\log_{10}$ PFU/mL at 7, 14, and 21 dpi, respectively. A significant difference in viral titer was found at 7 dpi vs 21 dpi ($p<0.05$). *Ae. aegypti* from Rio Grande that ingested 6 $\log_{10}$ PFU/mL showed a mean viral titer in legs of 1.82, 3.26 and 3.23 $\log_{10}$ PFU/mL at 7, 14, and 21 dpi, respectively. A significant increase ($p<0.05$) in viral titer of 1.34 and 1.37 $\log_{10}$ PFU/mL was detected at 14 and 21 dpi, respectively, in comparison to the viral titer obtained at 7 dpi.

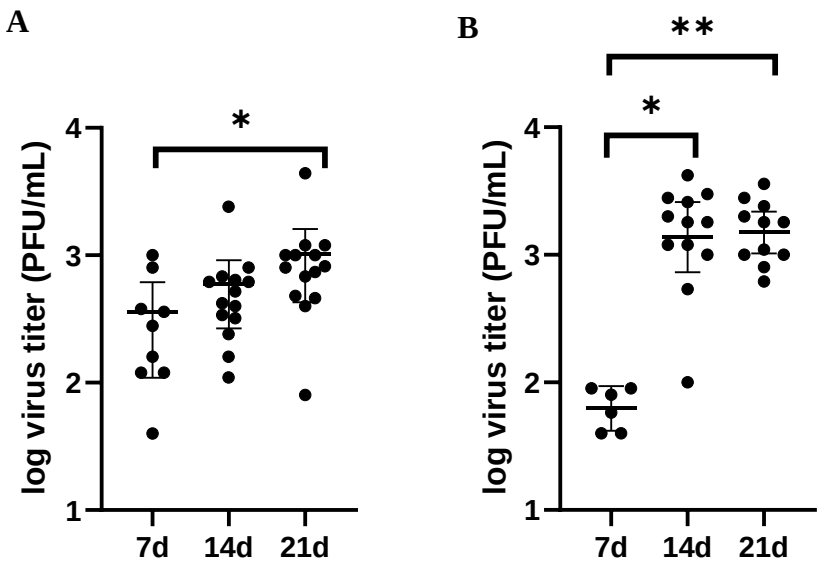

Figure 4.14 Virus titer in legs of infected *Ae. aegypti* from El Paso (**A**) and Rio Grande (**B**) after ingesting 6 $\log_{10}$ PFU/ mL of DENV-1.

Ae. aegypti from El Paso that ingested 4 $\log_{10}$ PFU/mL viral dose had a mean viral titer in the legs of 2.072 $\log_{10}$ PFU/mL at 7 dpi. Later, the viral titer increased significatively ($p<0.05$) to 2.75 and 2.81 $\log_{10}$ PFU/mL at 14, and 21 dpi, respectively (**Figure 4.15A**). In case of the Rio Grande *Ae. aegypti* mosquitoes that ingested a lower viral dose (4 $\log_{10}$ PFU/mL), one single

mosquito leg had a titer of 1.954 $\log_{10}$ PFU/mL at 7 dpi, while virus was not detected by plaque assays in the two infected mosquitoes at 14 dpi. Finally, a mean viral titer of 3.07 $\log_{10}$ PFU/mL was detected at 21 dpi. No statistical analysis was performed to support the increase of viral titer in Rio Grande mosquito legs at 21 dpi due to the limited number of samples.

As shown in **Figure 4.14** and **4.15**, viral titers in mosquito legs significantly increased through time in both the El Paso and Rio Grande *Ae. aegypti* strains and reached a plateau at 14 dpi after ingesting 4 or 6 $\log_{10}$ PFU/mL viral doses. These results indicated that DENV-1 was able to replicate in this tissue through time. These data were consistent with the 100% disseminated infection rates found in both *Ae. aegypti* strains reached at 14 dpi, meaning that DENV-1 found in legs was infectious and replicative.

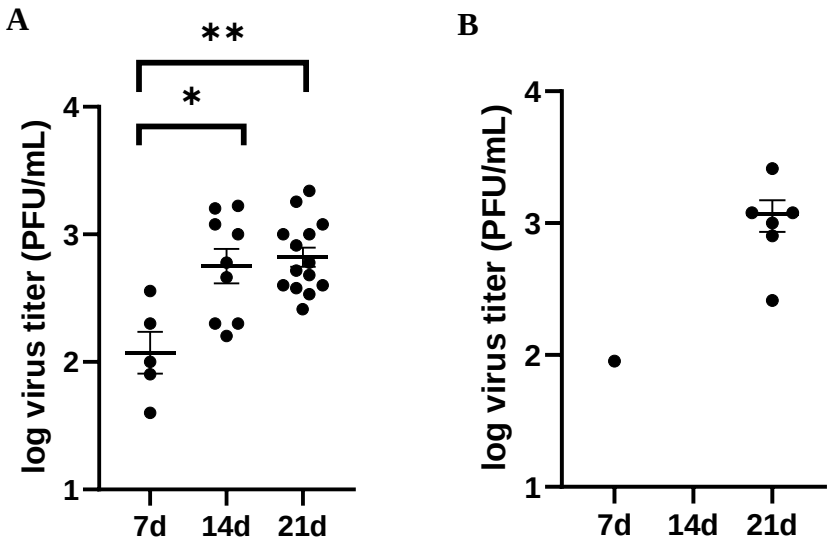

Figure 4.15. Virus titer in legs of infected *Ae. aegypti* from El Paso (**A**) and Rio Grande (**B**) after ingesting 4 $\log_{10}$ PFU/ mL of DENV-1.

4.3.2.9. DENV-1 dose does not affect the viral titers in *Ae. aegypti* mosquitos' legs

The comparison in the mean viral titers of *Ae. aegypti* mosquito legs after ingesting 4 or 6 log $_{10}$ PFU/mL of DENV-1 is shown in **Table 4.4.** Overall, no association was found between the viral dose and the mean legs titers in each time after infection ($p>0.05$), indicating than mean titers in the disseminated infected mosquitos' legs through time was not affected by a low or high viral dose exposure.

Table 4.4. The mean leg titers (log $_{10}$ PFU/mL) of *Ae aegypti* from El Paso and Rio Grande at 7, 14, and 21dpi after ingesting viral doses (4 log $_{10}$ PFU/mL vs 6 log$_{10}$ PFU/mL) of DENV-1.

ELP	Viral dose	6 log$_{10}$ PFU/mL	4 log$_{10}$ PFU/mL	
		Mean leg titers (PFU/mL)	Mean leg titers (PFU/mL)	
	7dpi	2.383	2.072	p>0.05
	14dpi	2.651	2.75	p>0.05
	21dpi	2.869	2.821	p>0.05
RIO	Viral dose	6 log$_{10}$ PFU/mL	4 log$_{10}$ PFU/mL	
		Mean leg titers (PFU/mL)	Mean leg titers (PFU/mL)	
	7dpi	1.797	1.954	p>0.05
	14dpi	3.139	ND	ND
	21dpi	3.176	2.982	p>0.05

ND = Not determined

4.3.2.10. Infected *Ae. aegypti* mosquitoes from the U.S. border did not differ from the leg viral titers

Furthermore, there was no difference in the mean leg viral titers of the mosquito strains from El Paso and Rio Grande after ingesting 6 log$_{10}$ PFU/mL viral dose at 7 and 21 dpi. (**Table 4.5**). A significant difference was observed at 14dpi, but then was not detected at 21 dpi. Similarly, both mosquito strains did not differ in the mean viral titer after ingesting a lower viral dose (4 log $_{10}$ PFU/mL) during the incubation period.

Table 4.5. The mean viral titers (log $_{10}$ PFU/mL) in the legs of the *Ae. aegypti* strains (ELP vs RIO) after ingesting 4 $\log_{10}$ PFU/mL or 6 $\log_{10}$ PFU/mL of DENV-1.

		ELP Mean leg titers (PFU/mL)	**RIO** Mean leg titers (PFU/mL)	
6 $\log_{10}$ PFU/mL	7dpi	2.383	1.934	p>0.05
	14dpi	2.651	3.139	*p<0.05
	21dpi	2.869	3.176	p>0.05
4 $\log_{10}$ PFU/mL	7dpi	2.072	1.954	p>0.05
	14dpi	2.75	ND	ND
	21dpi	2.821	2.982	p>0.05

Yellow highlighted box represent group where infectious saliva was detected.

Overall, the experimental results of both mosquito strains after infection with the Mexican DENV-1 isolate showed that the infectivity rate and the mean body titers in the mosquitoes were directly affected by the viral infectious dose (**Table 4.6**). In addition, the disseminated infection rates and the mean leg titer in the mosquitoes were mainly affected by the incubation period. Finally, transmission rates were low in both *Ae. aegypti* strains.

Table 4.6. Effect of biological variables (DENV-1 dose, incubation period, and U.S. border *Ae. aegypti* mosquito strains) in the vector competence indicators (infectivity and dissemination rates, mean body, and leg viral titers)

		Vector competence indicators				
		Infectivity rate	Mean body titer	Dissemination rate	Mean leg titer	Transmission rate
Variable	DENV-1 dose	**Affect**	**Affect**	No	No	ND
	Incubation period	No	No	**Affect**	**Affect**	ND
	U.S mosquito strains	No	No	No	No	ND

ND= Not determined

4.4. Discussion

4.4.1. DENV-1 isolation and characterization in a Mexican border community

The findings represent the first reported isolation of a DENV from a patient in Ciudad Juarez, Mexico, where previous serological evidence of DENV infection was reported among a human cohort (Palermo et al., 2019). The patient's clinical data was consistent with febrile illness with a DENV-1 viremia level of 2.69 $\log_{10}$ PFU/mL in the plasma. The isolation of DENV-1 in C6/36 cells was consistent with clinical criteria routinely used for DENV isolation from clinical samples (Jarman et al., 2011).

Viral replication of the DENV-1 isolate was higher in C6/36 cells than in Vero cells (**Figure 4.3**) and even though in vitro testing was performed, the findings suggested the ability of the DENV-1 isolate to replicate in it's the mosquito vector in nature. Furthermore, a limited number of passages were performed, restricting the adaptation of the DENV-1 isolate in the mosquito cell line.

Mexico has experienced several large outbreaks of dengue in the last two decades (2000-2019) causing more than 520 000 human cases (Arredondo-García et al., 2020) . Also, most of the dengue cases in the last 10 years have been reported from northern states of Mexico where DENV-1 and DENV-2 were the predominant serotypes. The DENV-1 isolate from this study clustered with other Mexican and Central American strains of DENV-1 belonging to the Genotype V. DENV-1 Genotype V have been circulating in Mexico since the late 1970s and has experienced several lineage replacements since its introduction causing a high degree of genetic variability (González-Durán et al., 2018).

Five amino acid changes in the DENV-1 isolate were unique in comparison with other Mexican DENV isolates (**Table 4.1**). Most of the changes involved amino acids with similar properties (E.g. NS1 (D139E), NS2 (K218R), and NS3(V332A, R338K)), that may not of have resulted in a viral protein dysfunction. Furthermore, four amino acid changes in the Ciudad Juarez strain were also found in other DENV-1 strains mainly from Asia. Briefly, NS1 139 D was changed to N in strains from China (GIZ-11), French Polynesia (BID-V2939), Hawaii (Haw03663), Japan (Mochizuki), Malaysia (P72-1244), and Thailand (606147 and ThD1-0081-82). NS2A 41 L was changed to F in Brazil (SJRP-2271), China (GIZ-11), and India (715393) strains. NS3 332 V was changed to A in Brazil (SJRP-2271), Gabon (Gabon2012), and Hawaii (Haw03663), and changed to I in the Thailand (606147) strain. Finally, NS3 338 R was changed to K in the French Polynesia (BID-V2939) strain.

The results suggested that the patient acquired DENV-1 infection in Ciudad Juarez because no travel history was reported before the onset of illness. Also, that DENV were endemic in this urban community is supported by the observation that 3 individuals seroconverted to DENV-1 infection during 2015 in Ciudad Juarez, and 2 of the 3 were positive for dengue IgM antibody (Palermo et al., 2019). Furthermore, a total of 10 human DENV-1 infections were reported in Ciudad Juarez in 2015 (DGE, 2015), thus documenting that the DENV-1 was endemic in this Mexican border urban community as a possible source for locally acquired DENV infections. A total of 12 dengue cases have been reported in Ciudad Juarez during the last 5 years, with DENV-1 being the most frequent cause of cases (n=11), and a single case was caused by DENV-2 during 2019 (DGE, 2016, 2017, 2019).

An alternative explanation for our findings regarding the human cases of dengue in Ciudad Juarez is the possibility of imported dengue cases resulting in secondary focal and

transient autochthonous transmission to cause one or more cases. The reported occurrence of sporadic outbreaks of dengue attributed to autochthonous transmission from 1980 to 2013 in Brownsville, Texas, and surrounding communities were associated with DENV infected travelers returning from visits to the bordering Mexican city of Matamoros during dengue epidemics in this community (Ramos et al., 2008; Rawlings et al., 1998; Thomas et al., 2016). Thus, a low number of secondary autochthonous cases may be acquired from returning viremic travelers as a source of infection and go undetected especially in areas without active surveillance programs and because asymptomatic or silent DENV infections are more difficult to detect than symptomatic cases (Chen et al., 1996; Fredericks & Fernandez-Sesma, 2014). Further longitudinal cohort studies in humans and mosquito surveillance are needed to obtain a better understanding of the dynamics of DENV transmission in this Mexican border community.

4.4.2. U.S border *Ae. aegypti* are susceptible and have efficient viral dissemination to DENV-1 Mexican isolate

This is the first vector competence study of *Ae. aegypti* mosquitoes from the U.S. border to a contemporary DENV-1 genotype V (Ciudad Juarez) strain isolated from the Mexican border region, highlighting the epidemiological importance for evaluating the risk of DENV transmission into the U.S.

DENV-1 infectivity rates in El Paso and Rio Grande *Ae. aegypti* strains were over 84% after ingesting a blood meal of 6 $\log_{10}$ PFU/mL and decreased to 40% to 57% after ingesting 4 $\log_{10}$ PFU/mL. As observed for DENV-2 (FSB 3381) and DENV-2 (328298) vector competence experiments in Chapter II, infectivity rates in both mosquito strains were significantly associated with the viral ingested dose. Furthermore, El Paso mosquitoes were more susceptible (MID_{50}< 4 $\log_{10}$ PFU/mL) to DENV-1 than Rio Grande mosquitoes (MID_{50}= 5.23 $\log_{10}$ PFU/mL) and this

feature was also observed for the DENV-2 (FSB 3381) strain in Chapter III. The remarkable susceptibility of El Paso *Ae. aegypti* mosquitoes for contemporary DENV strains [(DENV-2 (FSB 3381) and DENV-1(Cd Juarez)] suggested the limited effect of a midgut mosquito infection barrier to those DENV strains.

As previously reported with other DENV-2 strains in Chapter III, the titer of DENV per mosquito was 1 log $_{10}$ and 3 log $_{10}$ PFU/mL after ingesting 4 and 6 log $_{10}$ PFU/mL, respectively. In both El Paso and Rio Grande mosquito strains that ingested 6 log 10 PFU/mL, DENV-1 was able to replicate 1 log more (from 3 to 4 log PFU/mL) at 7 dpi and then the titer was sustained through the 21 days incubation period. Similarly, in mosquitoes that ingested 4 log $_{10}$, titer increased 2 log (from 1 to 3 log PFU/mL) at 7 dpi with no significant differences through the incubation period. DENV-1 mean titer detected in each infected *Ae. aegypti* mosquito body during the incubation period ranged from 4.2 to 4.6 log $_{10}$ PFU/mL after ingesting 6 log $_{10}$ dose was significantly higher than the 3.2 to 3.8 log $_{10}$ PFU/mL mean body titer found in mosquitoes that ingested 4 log $_{10}$ PFU/mL. Interestingly, those DENV-1 titers in mosquito bodies were higher than the 3.3 to 4.2 log $_{10}$ PFU/mL and 2.6 to 3.5 log $_{10}$ PFU/mL body titers found in mosquitoes that ingested DENV-2 (FSB 3381 or 328298) at 6 log $_{10}$ and 4 log $_{10}$ PFU/mL, respectively in Chapter III, indicating that DENV-1 replicates faster than any of the other DENV-2 strains evaluated in this dissertation. The replication of DENV-1 in mosquito bodies, based on infectivity assays (PFU), is the first report for this U.S. mosquito strains unlike some other vector competence studies that use viral RNA copies for measuring viral replication and did not represent infective virus (Calvez et al., 2017).

Also, even though less *Ae. aegypti* mosquitoes from El Paso and Rio Grande were infected with a low viral dose of 4 log $_{10}$ PFU/mL, DENV-1 was able to completely disseminate

(100%) in all the infected mosquitoes at 14 dpi, suggesting that there was no midgut escape barrier in the DENV-1 infected mosquitoes. This result is strikingly as in case of others DENV-2 (FSB 3381) and DENV-2 (328298) strains, highest dissemination rates were delayed to 21 dpi and were not able to reach 100% after ingesting a 4 $\log_{10}$ PFU/mL viral dose. This suggested that DENV-1 is efficient in disseminating in both *Ae. aegypti* strains. Also, even the viral titer of the body ~ 3.2-3.8 $\log_{10}$ PFU/mL was lower than ~ 4.2-4.6 $\log_{10}$ PFU/mL reported for 6 $\log_{10}$ PFU/mL ingested by the mosquitoes, it was enough to completely disseminate into the mosquito legs. Furthermore, disseminated infection rates and viral replication in legs increased with the incubation time as previously reported for other DENV-1 vector competence studies (Calvez et al., 2017; Gutiérrez-Bugallo et al., 2020). Finally, mean leg titers were not significantly affected by the viral dose of exposure or mosquito strains.

As was reported for other DENV-2 strain (FSB 3381 and 328298) in El Paso and Rio Grande mosquitoes, transmission rates based on the detection of DENV-1 in the saliva of disseminated mosquitoes was low in both *Ae. aegypti* (Rio Grande mosquitoes after ingesting 6 $\log_{10}$ PFU/mL viral dose (TR=3.23%) and in El Paso mosquitoes after ingesting 4 $\log_{10}$ PFU/mL of virus (TR=5.71%). These results suggested that a salivary gland barrier may be present in these U.S. border *Ae. aegypti* strains, which not only limited the release of DENV-2 strains into the saliva, but also the DENV-1 strain endemic in the Mexican border region (Palermo et al., 2021) . A recent study also detected a low transmission rate to another DENV-1 genotype V strain (Haiti 2012 isolate) in *Ae. aegyti* from Cuba (Gutiérrez-Bugallo et al., 2020)**.** On the other hand, the force salivation technique, and its methods for collecting saliva (blood, mineral oil, etc.), might have caused an underestimation of the potential of viral transmission causing a low transmission rate as reported previously (Gloria-Soria et al., 2022). An animal

model representing a true transmission method, where a susceptible animal is exposed to DENV-infected U.S. border *Ae aegypti* mosquitoes, would provide further evidence if mosquito barriers are present in these mosquito's strains.

Furthermore, even though *Ae. aegypti* is widely distributed in Ciudad Juarez, Mexico and serological evidence of DENV-1 infection locally acquired in Ciudad Juarez was previously reported (Palermo et al., 2019) , vector competence studies to DENV strains (including DENV-1) in *Ae. aegypti* from Ciudad Juarez are still lacking and would provide information if mosquito barriers to DENV are present in this *Ae. aegypti* population where DENV-1 appears to be endemic.

Finally, this is the first study that describes the interaction between *Ae. aegyti* and DENV in the U.S border. However, other factors for understanding the DENV transmission in this area needs further study as the mosquito life span, vector density, number of susceptible individuals, and contact index between host and vector. This last factor is critical and can be disrupted by the use of air conditioning, which have limited the indoor exposure of humans to *Ae. aegypti* and can be one of the causes of the limited transmission of DENV in the U.S. border (Reiter et al., 2003).

4.5. Recommended Future Directions

Further studies using animal models and contemporary DENV isolates in the vector competence of US/Mexican *Ae. aegypti* mosquitoes to DENV would provide critical information on the risk of DENV transmission in the US/Mexican border region. Finally, mosquito genetics studies of *Ae. aegypti* along the US/MX border would provide information in their genetic variability and clarify the role of the *Ae, aegypti* mosquito innate immune response to DENV.

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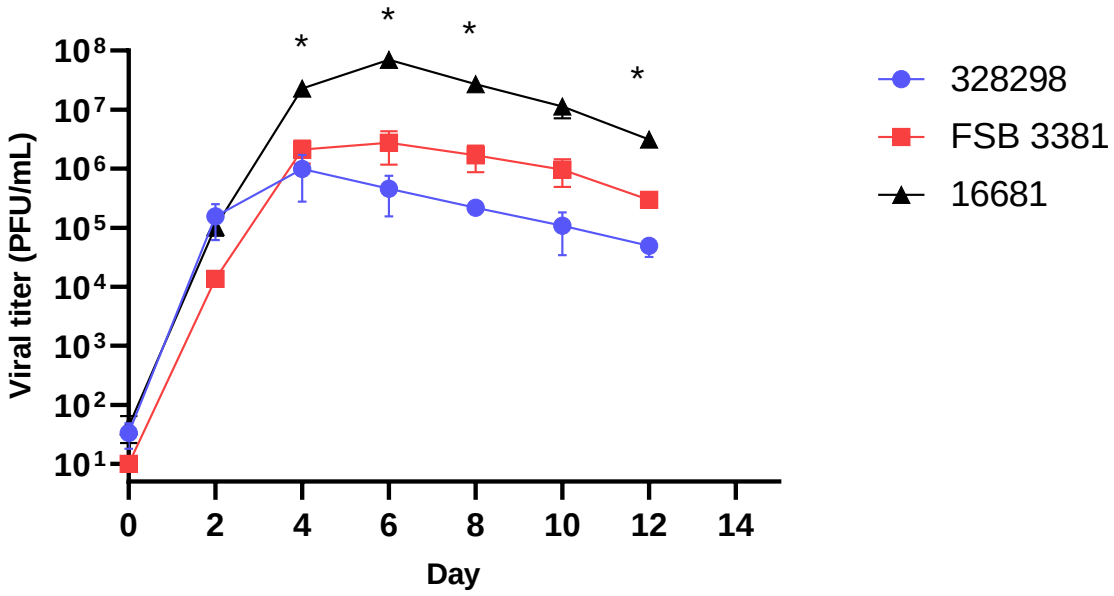
List of Appendices

Appendix 1. Replication curves of DENV-2 strains (FSB 3381, 328298, and 16681) in C6/36 cells.



(*) Significant differences ($p<0.05$) by Repeated Measures ANOVA in the viral titer between DENV-2 strains and the incubation period

Appendix 2. DENV strains for phylogenetic analysis. Strain names, accession numbers, and country and year of collection are included.

Tree Name	Collection Year	Collection Location	Strain Name	GenBank Accession
DENV1_Brazil_1990_Den1BR-90	1990	Brazil	Den1BR-90	AF226685.2
DENV1_Brazil_2010_13861-BR-PE-10	2010	Brazil	13861-BR-PE-10	JX669465.1
DENV1_Brazil_2014_SJRP-2271	2014	Brazil	SJRP-2271	KP188568.1
DENV1_China_2015_GZ-11	2015	China	GZ-11	KX225492.1
DENV1_Colombia_1998_BID-V3377	1998	Colombia	BID-V3377	GQ868560.1
DENV1_Colombia_1998_BID-V7290	1998	Colombia	BID-V7290	KJ189302.1
DENV1_Ecuador_2014_TD-00061-P	2014	Ecuador	TD-00061-P	KY474307.1
DENV1_Florida_2010_BOL-KW010	2010	Florida	BOL-KW010	JQ675358.1
DENV1_France_2014_Toulon-CNR-25329	2014	France	Toulon-CNR-25329	MF004384.1
DENV1_FrenchPolynesia_2001_BID-V2939	2001	French Polynesia	BID-V2939	FJ898448.1
DENV1_Gabon_2012_Gabon2012	2012	Gabon	Gabon2012	MG877557.1
DENV1_Hawaii_2001_Haw03663	2001	Hawaii	Haw03663	DQ672564.1
DENV1_India_1971_715393	1971	India	715393	JQ922546.1
DENV1_Japan_1943_Mochizuki	1943	Japan	Mochizuki	AB074760.1
DENV1_Malaysia_1972_P72-1244	1972	Malaysia	P72-1244	EF457905.1
DENV1_Mexico_2006_BID_V3658	2006	Mexico	BID_V3658	GU131958.1
DENV1_Mexico_2007_BID-V3673	2007	Mexico	BID-V3673	GU131964.1
DENV1_Mexico_2007_BID-V3679	2007	Mexico	BID-V3679	GU131966.1
DENV1_Mexico_2007_BID-V3709	2007	Mexico	BID-V3709	GQ868513.1
DENV1_Mexico_2007_BID-V3727	2007	Mexico	BID-V3727	GQ868521.1
DENV1_Mexico_2007_BID-V3739	2007	Mexico	BID-V3739	GQ868527.1
DENV1_Mexico_2007_BID-V7572	2007	Mexico	BID-V7572	KJ189317.1
DENV1_Mexico_2007_BID-V7589	2007	Mexico	BID-V7589	KJ189323.1
DENV1_Mexico_2007_BID-V7590	2007	Mexico	BID-V7590	KJ189324.1
DENV1_Mexico_2008_BID-V3758	2008	Mexico	BID-V3758	GQ868537.1
DENV1_Mexico_2011_BID-V7298	2011	Mexico	BID-V7298	KJ189306.1
DENV1_Mexico_2011_BID-V7624	2011	Mexico	BID-V7624	KJ189348.1
DENV1_Mexico_2011_BID-V7625	2011	Mexico	BID-V7625	KJ189349.1
DENV1_Mexico_2012_BID-V8195	2012	Mexico	BID-V8195	KJ189368.1
DENV1_Mexico_2015_CiudadJuarez	**2015**	**Mexico**	**Ciudad Juarez**	**MZ3432597**
DENV1_Nicaragua_2004_BID-V653	2004	Nicaragua	BID-V653	EU596501.1
DENV1_Nicaragua_2004_BID-V664	2004	Nicaragua	BID-V664	GQ199873.1
DENV1_Nicaragua_2005_BID-V1071	2005	Nicaragua	BID-V1071	EU482617.1
DENV1_Nicaragua_2005_BID-V534	2005	Nicaragua	BID-V534	JN819402.1
DENV1_Nicaragua_2005_BID-V536	2005	Nicaragua	BID-V536	FJ850113.1

DENV1_Nicaragua_2005_BID-V606	2005	Nicaragua	BID-V606	EU596504.1
DENV1_Nicaragua_2005_BID-V621	2005	Nicaragua	BID-V621	FJ410290.1
DENV1_Nicaragua_2005_BID-V629	2005	Nicaragua	BID-V629	FJ024485.1
DENV1_Nicaragua_2006_BID-V746	2006	Nicaragua	BID-V746	JN819403.1
DENV1_Nicaragua_2007_BID-V1223	2007	Nicaragua	BID-V1223	FJ898433.1
DENV1_Nicaragua_2008_BID-V2652	2008	Nicaragua	BID-V2652	GQ199859.1
DENV1_Nicaragua_2009_BID-V5504	2009	Nicaragua	BID-V5504	JF937635.1
DENV1_Nicaragua_2011_BID-V7672	2011	Nicaragua	BID-V7672	KF973460.1
DENV1_Nicaragua_2012_BID-V7651	2012	Nicaragua	BID-V7651	KF973456.1
DENV1_Nicaragua_2012_BID-V7655	2012	Nicaragua	BID-V7655	KF973458.1
DENV1_Nicaragua_2012_BID-V7690	2012	Nicaragua	BID-V7690	KF973472.1
DENV1_Nicaragua_2012_BID-V7691	2012	Nicaragua	BID-V7691	KF973473.1
DENV1_Nicaragua_2012_BID-V7692	2012	Nicaragua	BID-V7692	KF973474.1
DENV1_Nicaragua_2012_BID-V7696	2012	Nicaragua	BID-V7696	KF973475.1
DENV1_PuertoRico_1986_BID-V2097	1986	Puerto Rico	BID-V2097	FJ562106.1
DENV1_PuertoRico_1992_BID-V2136	1992	Puerto Rico	BID-V2136	FJ410186.1
DENV1_PuertoRico_1993_BID-V2134	1993	Puerto Rico	BID-V2134	FJ410185.1
DENV1_PuertoRico_1994_BID-V2127	1994	Puerto Rico	BID-V2127	FJ410179.1
DENV1_PuertoRico_1995_BID-V1744	1995	Puerto Rico	BID-V1744	FJ205875.1
DENV1_PuertoRico_1996_BID-V2138	1996	Puerto Rico	BID-V2138	FJ478457.1
DENV1_PuertoRico_2006_BID-V852	2006	Puerto Rico	BID-V852	EU482591.1
DENV1_PuertoRico_2012_BID-V7719	2012	Puerto Rico	BID-V7719	KJ189359.1
DENV1_Thailand_1960_606147	1960	Thailand	606147	JQ922547.1
DENV1_Thailand_1982_ThD1-0081-82	1982	Thailand	ThD1-0081-82	AY732481.1

DENV1_Venezuela_1997_BID-V2162	1997	Venezuela	BID-V2162	FJ639735.1
DENV1_Venezuela_1998_BID-V2168	1998	Venezuela	BID-V2168	FJ639740.1
DENV1_Venezuela_1998_BID-V2169	1998	Venezuela	BID-V2169	FJ639741.1
DENV1_Venezuela_1999_BID-V2171	1999	Venezuela	BID-V2171	FJ639743.1
DENV1_Venezuela_2000_BID-V3549	2000	Venezuela	BID-V3549	GU131833.1
DENV1_Venezuela_2004_BID-V2423	2004	Venezuela	BID-V2423	JN819425.1
DENV1_Venezuela_2006_VE-61006	2006	Venezuela	VE-61006	HQ332182.1
DENV1_Venezuela_2007_BID-V1134	2007	Venezuela	BID-V1134	EU482609.1
DENV1_Venezuela_2007_VE-61081	2007	Venezuela	VE-61081	HQ332183.1
DENV1_Venezuela_2008_BID-V2469	2008	Venezuela	BID-V2469	FJ850104.1
DENV2_PapuaNewGuinea_1944_NewGuineaC	1944	Papua New Guinea	New Guinea C	KM204118.1

Vita

Pedro Palermo was born in Lima, Peru in 1983. He graduated from San Marcos University in 2005 with a Bachelor's in Biology with a mention in Genetics and Cellular Biology. He worked as a Research Assistant in the Genomic Division at International Potato Center from 2006 to 2007. Also, he worked as an Associate Researcher at the Department of Mammalogy at the Natural History Museum in Lima, Peru. Later, he joined the Virology Department at the Naval Medical Research Unit 6 (NAMRU-6) from 2007 until 2012. During his time as Research Assistant at NAMRU-6, he pursued a Master's in Molecular Biology at the San Marcos University. He taught Virology and Molecular Systematics at the Cayetano Heredia and San Marcos Universities, respectively, and participated as oral speaker in several scientific conferences and symposiums. He participated in international training courses in Infectious diseases, Bioinformatics, and Genetics. In 2013, he joined the University of Texas at El Paso (UTEP) as a Biosafety level 3 Manager. Later, he was accepted to the Pathobiology program at UTEP in 2015. He participated in oral and poster presentations, published six manuscripts as first author, and is coauthor of other twelve manuscripts, and some others are accepted for publication. He received three recognition awards while working in NAMRU-6, and a local Funding Grant while he was a master's student in Peru. Finally, he obtained a DODSON funding award and travel awards from the UTEP Graduate School, and from the American Society of Tropical Medicine and Hygiene organization.

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