Dengue Risk Assessment

by

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Abstract

Dengue fever is the most prevalent vector borne disease in the world with over 3.6 billion people at risk and one billion new infections (including one million deaths) recorded annually. Risk assessments showing the impact of travel on the importation of dengue virus (DENV) are essential to understand the role of human travel in the spread of this mosquito-borne pathogen for which humans are the primary reservoir. Consequently, we calculated the exposure risk of United States (US) citizens traveling into Pan American countries where dengue (DEN) fever is endemic. The number of DEN cases in 51 Pan American countries was compared to the population of the same countries from 2001-2012 as a measure of exposure risk. Travel statistics (i.e. US travelers visiting the 51 Pan American countries) were analyzed and categorized by geographical region (i.e. North America, Central America, Andean, Southern Cone, Hispanic Caribbean and English, French and Dutch Caribbean). Travel patterns for US citizens were compared with subsequent DEN infections for each region visited. We show that US travelers visiting the Dominican Republic exhibited the highest number of imported DEN infections for the period of study. The Pan American country with the most DEN cases in its residents was Brazil (> 1 million reported cases in 2010). The numbers of DEN cases in Pan America

continues to rise as does international travel and the geographic range of potential DENV vectors. Hence, in order for DENV risk assessments to improve, we must analyze possible routes of entry for this pathogen. There is also an increased risk of introducing new DENV serotypes into naïve human populations. Regions where all four DENV serotypes are prevalent are at the highest risk for DHF and DSS. Underreporting and misdiagnosis remains an issue for calculating DENV transmission risk and this is discussed. In the second part of this study, commercially available blood was analyzed for life table characteristics and vector competence. Commercially available blood can be used as an alternative to live animals to maintain mosquito colonies and deliver infectious blood meals during research studies. However, the extent to which artificially delivered blood sources affect mosquito life table characteristics of *Aedes albopictus* and vector competence for dengue virus (DENV) is unknown. Consequently, we analyzed the extent to which two blood sources affected life table characteristics (i.e. fecundity, fertility, hatch rate, adult survival) and vector competence (infection, dissemination, transmission) of *Ae. albopictus* for DENV. Two types of blood (N = 40 mosquitoes/group) were tested at two extrinsic incubation temperatures for DENV-infected and –uninfected mosquitoes as follows: 1) defibrinated, 27°C; 2) citrate, 27°C; 3) defibrinated, 30°C; 4) citrate, 30°C. Fully engorged mosquitoes were transferred to individual cages containing an oviposition cup and substrate. The presence of eggs was observed daily and, if eggs were observed, the substrate was removed and the number of eggs was counted (fecundity) for each female. Eggs were allowed to hatch and larvae were counted (fertility) for each female. At 14 and 21 days post feeding, 15 mosquitoes were taken from each group and tested for DENV in bodies (infection), legs (dissemination), and saliva (transmission). Mosquitoes fed DENV-infected defibrinated blood showed significantly higher DENV body titer ($P = 0.034$) and fecundity ($P = 0.032$), as well as faster hatch time ($P =$

0.039) compared to mosquitoes fed DENV-infected citrated blood. Temperatures tested here did not ($P > 0.05$) affect any factor measured. No differences were observed in DENV leg titers between treatments. DENV transmission was observed in all groups 14 days post infection and was observed in all but the 30°C defibrinated blood group at the 21 day post infection time point. Infected mosquitoes showed higher fecundity than uninfected mosquitoes ($P = 0.001$); however, fertility was lower in infected compared to uninfected mosquitoes ($P = 0.001$). Eggs of DENV-infected mosquitoes hatched faster than the uninfected groups ($P = 0.005$). We expect the findings of this study to improve methods for mosquito colony propagation and inform research using artificial blood delivery methods to assess vector competence.

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LIST OF ABBREVIATIONS

ADP…….Adenosine Diphosphate

ATP……..…Adenosine Triphosphate

AMCA……. American Mosquito Control Association

ANOVA……...Analysis of Variance

CDC…….. Centers for Disease Control

cDNA…….....……Complementary Deoxyribonucleic Acid

DALY.....…...Disability-Adjusted Life Year

DDT…….. Dichlorodiphenyltrichloroethane

DENV…….Dengue Virus

DHF……...……Dengue Hemorrhagic Fever

DNA……Deoxyribonucleic Acid

DPI……Days Post Infection

DSS……..Dengue Shock Syndrome

EEEV……..….. Eastern Equine Encephalitis Virus

EPA……..…….. Environmental Protection Agency

IMS-Dengue…….Integrated Management Strategy for Dengue Prevention and Control

LACV……..…. La Crosse Virus

MEB……. Midgut Escape Barrier

MIB……... Midgut Infection Barrier

MMWR……Morbidity and Mortality Weekly Report

OTTI …….……Office of Travel and Tourism Industries

PAHO ……...Pan American Health Organization

PFU ……....Plaque Forming Units

qRT-PCR …....……Quantitative Reverse Transcription Polymerase Chain Reaction

RNA……...Ribonucleic Acid

SEB…….. Salivary Gland Escape Barrier

SIB…….Salivary Gland Infection Barrier

SLEV……..……...…..…. St. Louis encephalitis virus

US……...……. United States

WHO……. World Health Organization

WNV…….…..… West Nile Virus

WTO……... World Tourism Organization

Chapter 1 – Introduction and Purpose

Vector-borne diseases account for 17% of the burden caused by all infectious diseases globally, with one billion new infections (including one million deaths) recorded annually (World Health Organization [WHO, 2014]). Historically, diseases such as dengue (DEN), malaria, yellow fever, filariasis, plague, louse-borne typhus and other vector-borne diseases were responsible for more human disease and death between the 17th and 20th centuries than all other causes combined (Gubler, 1998). Dengue is the most prevalent vector borne human illness (Durbin, 2011, Ross 2010) and there were > 2.5 billion people at risk for DEN infection in 2002 (Gubler, 2002) (Figure 1). In recent years, the number of "at risk" people has increased to *ca.* 3.6 billion (Halstead, 2007; Tolle, 2009; Meltzer and Schwartz, 2009; Halstead, 2013). As of 2002, 50-100 million new DEN infections were reported each year, including *ca*. 500,000 severe cases/year (Holmes and Twiddy, 2002; Ligon, 2004) and ≥ 21,000 deaths/year (Holmes and Twiddy, 2002; Halstead, 2007; Durbin, 2011; Pye, 2012). However, recent studies show 390-400 million DEN cases/year (Bhatt, 2013; Cross, 2013; Yacoub and Wills, 2014). Globalization and climate change (e.g. temperature increase) may facilitate the increased geographic range of vectors that transmit dengue virus (DENV; *Flaviviridae: Flavivirus*), the pathogen that causes DEN (Gratz, 1999; Gubler, 2002; Holmes and Twiddy, 2002; Pye, 2012; Tantawichien, 2012).

Dengue virus is transmitted by mosquitoes (primarily *Aedes aegypti* L. and *Ae. albopictus* (Skuse) to humans (epidemic cycle) and primates (sylvatic cycle) (Weaver, 2010; Holmes and Twiddy, 2002). Dengue virus has four distinct serotypes (DENV-1, DENV-2, DENV-3, DENV-4) (Gubler, 2002; Simmons 2012) and multiple genotypes, named by geographical location (Costa, 2011). Most people infected with only one serotype do not experience symptoms and lifelong immunity to that serotype is achieved (Mangold and Reynolds, 2013; WHO, 2014);

however, infection with multiple serotypes may lead to severe DEN [i.e. dengue hemorrhagic fever (DHF), dengue shock syndrome (DSS)] (Holmes and Twiddy, 2002; Durbin, 2011; Whitehorn and Farrar, 2011). Severe DEN is most common when secondary infection with a different serotype occurs (Yacoub and Wills, 2014) and severe symptoms manifest themselves whenever the viral load is in decline (Guzman et al., 2000). There is temporary immunity (two to three months) to all serotypes after initial infection with one serotype (Halstead, 2007; Rothman, 2013; WHO, 2014). There are no vaccines or antiviral treatments available to protect people from DENV (Meltzer and Schwartz, 2009; Yacoub and Wills, 2014), hence vector control is essential (Durbin, 2011; Dick, 2012).

In the Americas, outbreaks of DEN occur in an endemo-epidemic (i.e. the virus is endemic but occasionally becomes epidemic) pattern with outbreaks every three to five years (Dick, 2012). The circulation of multiple serotypes commonly causes severe epidemics (Gubler, 2002; Ross, 2010) and this phenomenon has continued to increase (Lorono-Pino et al., 1999, Gubler, 2004; Guzman and Harris, 2015). Outbreaks of DEN have been well documented since the early 1950s and are increasing, causing economic burdens and proving that preventative vector control measures should be taken (Holmes and Twiddy, 2002; Gubler, 2002). Since the late 1970s, DENV has become endemic in the Americas, resulting in millions of cases and thousands of deaths (Gubler, 1989). Millions of dollars are spent annually on vector control to prevent and control vector borne disease in the US and, despite this, revenue has been lost due to stagnating tourism and decreased workforces (Knudsen and Slooff, 1992).

Dengue emergence/reemergence is especially concerning in areas with competent vectors (Richards et al., 2012). A better understanding of DENV-vector interactions will enhance risk

assessments of DEN expansion. Previous studies have explored the impact of DENV on life table characteristics (i.e. fertility, fecundity, lifespan, hatch time, etc.) in *Aedes aegypti* (primarily subtropical/tropical) (Sim and Dimopoulos, 2010; Carrington et al., 2013); however, little research has been conducted using *Ae. albopictus* (primarily temperate regions), another efficient vector of DENV. Research on factors influencing the ability of *Ae. albopictus* to transmit DENV (i.e. vector competence) will improve predictions for the risk of DEN expansion, hence mitigating outbreaks. The establishment of *Ae. albopictus* in previously uninfested regions shows that few areas with ecological conditions suitable for the vector's survival are secure from its introduction and establishment (Gratz, 1999). Durbin (2012) suggests that mapping historical data on traveler-imported DEN cases may improve risk assessments. Prevention and control of DF and DHF is urgent due to the increasing geographic distribution of vectors and disease incidence over the past 20 years (Monath, 1994).

The increase in global travel (WTO, 2012) coupled with the geographic expansion of DENV and one of its primary vectors, *Ae. albopictus*, is the primary focus of this study. Consequently, we aim to: 1) Determine how human travel between Pan American countries and the US affects the spread of DENV, and 2) Assess the extent to which different blood sources used in laboratory vector competence studies impact life table characteristics and vector competence of *Ae. albopictus*.

Chapter 2 – Literature Review

History of dengue in the Americas

There is controversy over where DENV originated. The earliest record was found in a Chinese medical encyclopedia in 992 A.D. (Gubler, 1998). An African origin of the virus was suggested by Gaunt et al. (2001) because of the number of other mosquito-borne flaviviruses that originally circulated exclusively in Africa. *Aedes aegypti* also likely originated in Africa (Holmes and Twiddy, 2002). However, the same study discusses that all four DENV serotypes have been documented in monkeys and humans from Asia. The first DEN-like epidemics were reported in 1635 in Martinique and Guadeloupe and in Panama in 1699; however, due to the lack of epidemiological knowledge at the time, it is uncertain whether the diagnoses were correct (Dick, 2012). By the 1700s, a disease resembling DEN caused epidemics in Asia and the Americas. The first well documented outbreak of DHF occurred in Manila, Philippines in 1953-54, followed by an outbreak in Bangkok, Thailand in 1958 (Holmes and Twiddy, 2002).

During the 18^{th}-20^{th} centuries, DEN outbreaks occurred in coastal cities of the Caribbean, North, Central, and South America, and were related to commercial shipping activities (Gubler, 2002; Guzman, 2003). After a yellow fever epidemic in Rio de Janeiro, Brazil in 1928 and the discovery of jungle yellow fever in 1932, scientists realized that the eradication of *Ae. aegypti*, the primary vector of DENV in tropical regions, could protect people from yellow fever and DEN (Gubler, 1998; Gratz, 1999; Dick, 2012). Dengue research started in 1943 when DENV was isolated for the first time (Severo, 1955). Movement of military troops facilitated DENV spread during World War II (WWII) (Ligon, 2004; Tavodova, 2012), i.e. troops became infected while deployed in Southeast Asia and returned home to the Americas (Ligon, 2004).

After WWII, tropical urbanization and international trade (WHO, 1971) increased. Populations of *Ae. aegypti* and *Ae. albopictus* spread throughout both hemispheres, increasing abundance and ranges of the vectors (Gubler, 2002; Holmes and Twiddy, 2002; Weaver, 2010). Most medical diagnoses were based on symptoms, while serology was used to confirm the infection for research (not treatment) purposes (Dick, 2012). The government of Brazil collaborated with the Rockefeller Foundation of the US to eliminate *Ae. aegypti* from Brazil (Severo, 1955). Other countries followed, including Bolivia, Colombia, Ecuador, Paraguay, and Peru (Dick, 2012). Abandoned water-holding containers were destroyed, pesticide fumigation was performed and mosquito surveillance increased to ensure *Ae. aegypti* populations did not recolonize treated areas (Dick, 2012). With the advent of dichlorodiphenyltrichloroethane (DDT), it was relatively easy for mosquito control personnel to suppress vector populations (PAHO, 1947). By 1962, 18 continental countries and many Caribbean islands had eradicated *Ae. aegypti* (Gratz, 1996; PAHO, 1997; Gratz, 1999). However, over the next 10 years, only three more countries eradicated *Ae. aegypti* as the program deteriorated due to the loss of political interest, lack of support from the health sector, and insufficient community participation (Gubler, 2002; Ross, 2010). Surveillance and control declined, allowing small *Ae. aegypti* re-infestations to occur (Ross, 2010; Dick, 2012). Rapidly growing urban centers, expansion of international and domestic travel, and insufficient environmental sanitation favored re-infestation by *Ae. aegypti* (PAHO, 1997; Gubler 1998; Gratz, 1999; Gubler, 2002; Ross, 2010). In < 20 years, the American tropics and the Pacific islands went from not having DEN to having a severe DEN problem by 1998 (Gubler, 1998). In Asia, proper mosquito control had never been achieved and severe forms of DEN emerged after WWII (Gubler, 1998).

Before 1970, only nine countries had experienced epidemics of DHF; however, by 1996, that number had increased to 102 countries (Gubler, 2002; Dorji, 2009). Global numbers of DEN cases increased during the 1970s-1980s and continued to increase in subsequent years (Gubler, 1998; Gratz, 1999; Gubler, 2002). In 1977, DENV-1 was detected for the first time in Cuba and serological surveys showed that more than 44% of humans were infected (Guzman et al., 2000; Halstead, 2007). By 1981, nearly all (98%) DHF and DSS cases in children and adults were associated with infection by a second DENV serotype (Diaz, 1988; Rothman, 2013). In 1981, Cuba had an outbreak with 344,203 DEN cases and 10,312 DHF cases, including 158 deaths (Kouri, 1981, Gratz, 1999). The first case report of infections with multiple DENV serotypes occurred in 1982 in Puerto Rico (Gubler, 1985). In 1999, infection with three serotypes was found in two patients from Indonesia and one from Mexico (Chinnawirotpisan, 2008). From 2000-2010, all four DENV serotypes were found in the Americas, causing the highest record of cases seen to date (Dick, 2012). Ecuador had 22,937 cases in 2000 with both DENV-2 and DENV-3 circulating. In Paraguay, approximately 25,000 cases of DENV-1 were reported in 2000 (PAHO, 2010). Peru had its worst outbreak in 2001 with 23,329 cases when all four serotypes were documented (Vargas, 2005). In 2002, there was a record number of *ca.* one million cases throughout Pan America (Dick, 2012) and Brazil accounted for over 75% of these cases (Nogueira, 2005). Costa Rica experienced 35,000 cases in 2005 (Gonzalez, 2007; Dick, 2012). Paraguay saw a mortality rate of 11.5% in 2006 with over 28,000 cases reported (Dick, 2012). In 2007, DENV-1, DENV-2 and DENV-3 circulated through Brazil causing 559,954 DEN cases, followed by 734,384 cases in 2008 (Saude, 2007; Saude, 2008). Bolivia experienced an outbreak with more than 84,000 cases and a mortality rate of 12.6% in 2009 (PAHO, 2010). DENV-1 caused more than 26,000 cases in Argentina in 2009. Over 250,000 cases were reported

in Mexico in 2009 and Nicaragua had an outbreak of 17,140 cases in the same year (Dick, 2012). Another Pan American outbreak occurred in 2010 with 1.7 million cases and 1,855 deaths. All 36 patients experiencing DHF in Puerto Rico in 2010 died (PAHO, 2010).

Global Burden of DENV

Dengue virus is endemic in many tropical and sub-tropical regions and is a leading cause of post-travel fever (Wilder-Smith, 2007). The risk of DEN is thought to be as high as malaria and higher than typhoid or hepatitis A (Wilder-Smith, 2007). With nearly half the world's population living in DENV-endemic regions, most pediatric hospitalizations in these regions are due to DEN (Tolle, 2009; WHO, 2005). To determine the global burden and impact on morbidity and mortality of a disease on a population, the World Bank developed the 'disability-adjusted life years' (DALYs) index, which defines the impact of the morbidity and mortality of a disease on the population (Murray and Lopez, 1996; Meltzer, et al., 1998; Gubler, 2002). Dengue costs hundreds of thousands of DALYs at 427 DALYs/million population (Gonzalez, 2011). In the US, each person hospitalized from DEN accumulates *ca.* $17,803 in medical costs and less severe cases cost *ca*. $1,610. A study reports the economic cost of DEN cases in the Philippines in 2012 was $345 million ($3.26 per capita), a 54% increase from 2010 (Edillo et al., 2015). When the same study estimates underreported DEN cases, healthcare costs equal the annual income of 242,000 Philippine people. The median cost of DENV-infected patient treatment throughout Pan America is $1,227/case (Shephard, 2011). Costs vary substantially between countries due to the value of the dollar and the variance of direct costs (Gonzalez, 2011), i.e. regional differences in medical costs. Proper health care facilities are not accessible to all DEN patients, hence underreporting occurs (Dick, 2012). Most DENV endemic countries have poor surveillance of DF/DHF/DSS. Research has shown that the impact of DF/DHF is on the same

order of magnitude as infectious diseases such as malaria, hepatitis, tuberculosis and bacterial meningitis (Meltzer et al., 1998). Over the past 30 years, DEN has become a major cause of morbidity and mortality in children throughout the tropics (Tolle, 2009). It has generated public attention and is feared by the general public, placing significant pressure on the health care system, especially during epidemics (WHO, 2005).

One of the most important factors for prevention and control of DEN is surveillance (Ligon, 2004). An analysis of the costs associated with arboviral surveillance budget cuts in Australia shows that delaying control responses (e.g. application of pesticides, source reduction) causes an exponential increase in healthcare costs and number of human cases (Vazquez-Prokopec et al., 2010). The use of proactive surveillance strategies (mosquito collection/testing along with human health records to determine risk assessment) for DEN outbreaks in Australia in 2003 and 2009 attributed to less money spent on reacting to outbreaks ($115,000 and $1.1 million spent on reacting, respectively) compared to a projected cost of $13 million and $382 million if these strategies would have been delayed 4-6 weeks (Vazquez-Prokopec et al., 2010; Queensland Government, 2011). This shows that a proactive surveillance program may lessen the economic burden of disease outbreaks and prevent a significant number of human cases.

The identification of high risk areas can assist vector control management by providing information of where funding and control measures would have the most impact (Hu, 2010). In most areas endemic for DEN, transmission of DENV varies seasonally and is highest during the rainy seasons (Wichmann and Jelinek, 2004). In third world countries, the close proximity and poor construction of houses and buildings, low access to health services and education and the

use of natural ventilation (i.e. no window screens) instead of air conditioning, facilitates the transmission of DENV (Gubler, 2002; Gonzalez, 2011).

It is expensive to manage vectors of DENV. Ultra low volume (ULV) insecticide sprays have been used for years to control epidemics (Gratz, 1991). This is a very visible application; however, combined with the government stating that they have the epidemic under control, the public may feel a false sense of security, hence leading to reductions of personal vector control around their own homes (e.g. dumping water-holding containers) which may perpetuate the epidemic cycle (Gubler, 2002). Other measures can be taken to reduce the spread of DENV including the use of repellent, which should be applied to exposed skin or clothing and should contain DEET or picaridin (Pye, 2012). However, consumers should be aware that products with less than 10% active ingredient will likely only offer limited protection (one to two hours) and concentrations above 50% offer no added protection (National Travel Health Network and Centre [NaTHNaC], 2010). The larval stage of mosquito development is the primary target for mosquito control strategies including introduction of larval predators and larvicides and oviposition habitat elimination (removal of standing water) (Tolle, 2009). Window screens, air conditioning, mosquito coils and bed nets are advised (Wichmann and Jelinek, 2004; NaTHNaC, 2010). While there is no vaccine (Whitehorn and Farrar, 2011; Meltzer and Schwartz, 2009), the best method of protection is prevention of mosquito bites with products like DEET as well as tipping over any water-holding containers (Mangold and Reynolds, 2013).

Evolution of the Virus

Dengue virus is a positive sense single stranded RNA virus (Holmes and Twiddy, 2002; Ross, 2010; Simmons, 2012). RNA viruses exhibit genetic variability due to high mutation rates

associated with RNA-dependent RNA polymerase (Drake and Holland, 1999). This high mutation rate increases virus biodiversity and potentially increases disease severity and difficulties with vaccine efficacy (Holmes and Burch, 2012). Unlike most other flaviviruses, DENV utilizes humans as a reservoir and amplification host. There are four genotypes of DENV-2 (the most virulent serotype) (Martina, Koraka and Osterhaus, 2009; Afreen et al., 2014). The two most notable genotypes of DENV-2 are: 1) American; endemic in the Western Hemisphere and South Pacific islands for five decades (first isolated in Trinidad in 1953) (Dick, 2012), and 2) Southeast Asian; first detected in the Americas (Cuba) in 1981. The Southeast Asian genotype has displaced the American genotype and is the more virulent form (Anderson, 2006).

Each DENV serotype is phylogenetically distinct (Kuno et al., 1998; Holmes and Twiddy, 2002). Phylogenies show the same branching order among the four DENV serotypes, with DENV-4 diverging first. The next serotype to evolve was DENV-2 followed by a split between DENV-1 and DENV-3 (Holmes and Twiddy, 2002). Serotypes may have originated independently due to geographic (allopatric) or ecologic partitioning in different primate populations (Holmes and Twiddy, 2002). Another hypothesis is that DENVs evolved together (sympatric), facilitating viral transmission through antibody-dependent enhancement (Ferguson, Anderson and Gupta, 1999; Tolle, 2009).

Diagnosis of DEN is primarily carried out via Immunoglobulin M (IgM), an antibody produced by B cells (Whitehorn and Farrar, 2011). Nonstructural protein 1 (NS1) in DENV can be detected in the first few days of infection. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a sensitive method for DENV detection, but is costly and required trained

personnel, hence, it is impractical for widespread use. A rapid sensitive test that detects NS1, the most common antibody [Immunoglobulin G (IgG)], and the largest antibody (IgM), allowing DENV identification throughout the intrinsic incubation period is needed (Whitehorn and Farrar, 2011). Patients with DSS exhibit higher IgG compared to patients with DHF and DF (Khan et al., 2013). Khan et al. (2013) shows that most (72.7%) DSS patients required medical intervention compared to DHF (25.4%) and DF (6.1%) patients. Other diagnostic tests include DENV isolation in cell culture, but this is expensive, time consuming, and cannot differentiate between serotypes (Guzman and Kouri, 1996; Mangold and Reynolds, 2013).

Dengue symptoms in vertebrate hosts

There are a range of symptoms associated with DEN infection (Holmes and Twiddy, 2002). The intrinsic incubation period (time between infection and symptoms) is three to seven days (Tavodova, 2012). Some patients are asymptomatic, while others experience flu-like symptoms (i.e. DF) (Simmons, 2012). Symptomatic patients may experience fever (>38.5^{0}C), headache, vomiting, myalgia, irritability, lethargy, abdominal pain, joint pain, and/or macular rash (Reiter, 2010; CDC, 2012; Simmons, 2012; WHO, 2012). The duration of symptoms is three to seven days and most patients will recover (Yacoub and Wills, 2014). In some cases, infections can progress to DHF or DSS (Gubler, 2002; Simmons, 2012). Some patients experience increased vascular permeability (Tantawichien, 2012), hemoconcentration, hypoproteinemia, pleural effusions and ascites (Simmons, 2012; Yacoub and Wills, 2014). The symptoms of plasma leakage are hypotension, tachycardia, cold extremities and restlessness. Mortality of patients experiencing severe forms of DEN varies between 10 – 20% (Whitehorn and Farrar, 2011; Travodova, 2012). Globally, > 500,000 DEN-related cases/year require hospitalization (Holmes and Twiddy, 2002). If early treatment is received with aggressive fluid

replacement therapy, patient mortality is reduced to < 1% (Ligon, 2004; Whitehorn and Farrar, 2011; Tavodova, 2012).

In Asia, all four serotypes exist in sylvatic transmission with primates (Tavodova, 2012). Other mosquito species such as *Aedes polynesiensis* Marks, *Aedes pseudoscutellaris* Theobald, *Aedes malayensis* Colless, *Aedes furcifer* Edwards (Jupp and Kemp, 1993), *Aedes taylori* Edwards, *Aedes luteocephalus* Newstead and *Aedes cooki* Belkin can transmit DENV in the sylvatic cycle (Rosen et al., 1985). The majority of primates do not exhibit symptoms after infection with DENV (Tavodova, 2012; Vasilakis et al., 2012; Clark et al., 2013). Monkeys in the *Macaca* and *Presbytis* genera are most commonly infected with DENV in Asia (Homes and Twiddy, 2002). Laboratory studies performed with Rhesus macaque (*Macaca* spp.) monkeys (South, Central, and Southeast Asia) indicate positive tests for DENV in regional lymph nodes 24 hours post infection (Rothman, 2013; Marchette et al., 2013). Studies performed with *Macaca fascicularis* Raffles monkeys (Southeast Asia) illustrates infection in sera by RT-PCR and virus isolation from all monkeys tested three days post infection (Bernardo et al., 2008). These species of *Macaca* did not exhibit symptoms of infection (Angsubhakorn et al., 1994; Bernardo et al., 2008).

Of the locally acquired human DEN cases in Florida in 2009, fever was reported by all 28 patients; rash, myalgia, arthralgia, eye pain and headache were also commonly experienced (MMWR, 2010). Six Floridian patients had bleeding, including four patients with bloody urine, one with vaginal bleeding (menorrhagia), and one with nosebleeds (MMWR, 2010; Tantawichien, 2012). Menorrhagia is common (25%) among female adults with DF/DHF (Carles, Peiffer and Talarmin, 1999; Thaithumyanon et al., 1994; Tantawichien, 2012). Increased

liver enzymes have also been found during DEN infection (Kuo et al., 1992; Kalayanarooj et al., 2007). Liver disease and encephalopathy are correlated in patients with DF/DHF, hence causing altered consciousness, seizures and coma due to encephalitis (Solomon et al., 2000). Autopsies show DENV replication can occur in bone marrow, midbrain, spleen, liver, kidney, and lymph nodes and the antigen can be found in the lymphoid cells of the spleen and thymus, necrotic liver tissue, thymus, lymph nodes, liver, spleen, lung and in skin cells (Perng, 2011).

Epidemiologic studies have identified extremes in age (Hammond, 2005; Lee, 2006), high and low body mass index (Yacoub and Wills, 2014; Kalayanarooj, 2005), secondary infection (Guzman, et al. 2013), female sex, pregnancy (Machado, 2013), Caucasian race, AB blood type (Khan et al., 2013), virus strain, and genetic variants as risk factors for severe DEN and/or complications (Simmons, 2012). There is a significantly higher percentage of male patients during epidemics; however, females have also been associated with severe disease (Anders et al., 2011; Huy et al., 2013; Khan, 2013). In Asia, DF and DHF predominantly affect children (thought to be due to their developing immune systems and increased time spent outside), while in the Americas, all age groups are equally affected (Guzman, 2003; Tantawichien, 2012). Race plays a significant role in infection as DEN infections were found to be more prevalent in the Caucasian race when compared to African American and Asian (Bravo, Guzman and Kouri, 1987). Interestingly, DHF and DSS are more common in well-nourished children than in malnourished children, likely due to lower levels of the monocyte Tumor Necrosis Factor – Alpha (TNF-α) in malnourished children, causing a lesser immune response (Rothman, 2013). It has been hypothesized that malnutrition can suppress the cell-mediated immune responses found to be associated with severe DEN (Maron et al., 2010; Rothman, 2013;

Daniel et al., 2014). This is due to the need for a strong inflammatory response for severe forms of DHF and DSS (Halstead, Chow and Marchette, 1973; Lei et al., 2001; Maron et al., 2010).

Despite the known symptoms, the majority of DEN infections are asymptomatic, making it difficult to diagnose or determine the extent to which it affects public health because they are diagnosed as non-differential fevers of unknown etiology, masking the prevalence of DEN (MMWR, 2010). Most imported DEN cases are never reported due to the association with flu-like illness (Gubler, 1998). This makes it virtually impossible for mosquito control programs to be effective, because information on infection rates is essential to take actions such as targeted mosquito control (MMWR, 2010). The ability to diagnose DEN in the early stages, combined with the ability to predict the patients who are more at risk for developing severe disease would significantly aid clinical management of DEN (Whitehorn and Farrar, 2011). If regions are unable to manage the disease, there is an increase in mortality due to overloading of clinics and hospitals, overworked medical staff, and sub-standard care for patients with severe DEN (Gubler, 2002; Tolle, 2009).

Mosquitoes

Mosquitoes are responsible for more human suffering worldwide than any other organism (AMCA, 2011). There are 3,500 mosquito species found throughout the world (Tolle, 2009). The two main DENV vectors are *Ae. aegypti* and *Ae. albopictus*. *Aedes aegypti* was not originally a domestic mosquito, but adapted to peridomestic settings in Africa (Weaver, 2010) and is now commonly found in urban areas in tropical climates (Maciel-de-Freitas et al., 2013). *Aedes aegypti* was likely transported into the Americas via ships (Gubler, 2002; Weaver, 2010). Female *Ae. aegypti* use artificial water-holding containers (e.g. buckets, bird baths, water storage barrels,

tires) as oviposition sites (Vezzani and Carbajo, 2008; Holmes and Twiddy, 2002; Ligon, 2004) and preferentially feed on human blood (anthropophilic) as a protein source for egg development and energy (Briegel, 1985; Harrington, Edman and Scott, 2001; Tolle, 2009; Weaver, 2010). Larval development time depends on environmental conditions including nutrients, day length, and temperature, i.e. most larvae develop into adults in approximately one week (Tolle, 2009). In temperate zones, larvae may overwinter (White, Cook and Zumla, 2004). *Aedes albopictus* is a day biting species that was originally a forest species found in Asia, which began a geographic expansion in the 1980s and is still expanding today (Lambrechts, 2010). *Aedes albopictus* was introduced into Texas in 1985 in a shipment of tires from Asia (Hawley et al., 1987; Gubler, 1998; Benedict et al., 2007) and introduced into California in 2001 in a shipment of "Lucky Bamboo" imported from China (Kuno, 2012). This species can survive in both temperate and tropical regions (Rezza, 2012). *Aedes albopictus* blood feeds on humans and domestic animals and oviposits in both natural and artificial containers that hold water (Lambrechts, 2010). Natural containers include bromeliads, tree-holes, and bamboo stumps (Marques, Santos and Forattini, 2001). Temperatures above 32.2°C reduce longevity of *Ae. albopictus*, while temperatures 15.5°C-22.2°C increase lifespan (Kuno, 2012). *Aedes albopictus* eggs can diapause, allowing the species to survive/overwinter during cold temperatures in which adult mosquitoes could not survive (Pumpuni, Knepler and Craig, 1992; Hanson and Craig, 1995; Brady et al., 2013). *Aedes aegypti* eggs cannot survive such extreme temperatures (Fischer et al., 2011). The eggs of both potential vectors are desiccation-resistant and can remain viable for over a year out of water (Liu et al., 2011; Rezza, 2012).

Even though *Ae. albopictus* can survive in both rural and urban areas, *Ae. aegypti* is considered a better vector of DENV (Lambrechts, 2010; Rezza, 2012). Some explanations of this

include: 1) *Ae. aegypti* is adapted to urban environments while *Ae. albopictus* prefers suburban or rural settings due to increased vegetation density (Lambrechts, 2010), 2) *Ae. aegypti* prefers to bite humans over other hosts (Scott et al., 1993; Harrington, Edman and Scott, 2001; Ponlawat and Harrington, 2005; Lambrechts, 2010) and is a competent vector of DENV, yellow fever virus, chikungunya virus, Zika virus and West Nile virus (WNV) while *Ae. albopictus* is an opportunistic feeder and has been shown to vector > 22 pathogens (Tiawsirisup, 2003; Gratz, 2004; Vega-Rua et al., 2013), 3) *Ae. aegypti* stops feeding at the slightest movement and will either return to the same or a different host to continue feeding (Gubler, 1998; MMWR, 2010) and will feed exclusively on humans if given the opportunity (Ponlawat and Harrington, 2005). *Aedes aegypti* may feed on multiple human hosts during a single gonotrophic cycle, hence increasing its probability of becoming infected and transmitting a pathogen to another host (Weaver, 2010; Rezza, 2012). It is common to find members of the same household infected with DF within a 36 hour period, possibly infected by the same mosquito (Gubler, 1998; Mangold and Reynolds, 2013).

Dengue virus can invade the mosquito's brain tissues, which can modify mosquito metabolism and physiology, hence changing life-history traits involved in vector competence (Putnam and Scott, 1995; Maciel-de-Freitas et al., 2013). Dengue virus-infected *Aedes aegypti* feed on multiple hosts more frequently compared to uninfected mosquitoes due to virus-induced increase (up to 50%) in locomotor activity (Lima-Camara et al., 2011). This may increase bite rate and potentially increases the chance of mosquitoes infecting naïve hosts (Lima-Camara et al., 2011). The lifespan of *Ae. aegypti* is *ca.* 40 days (MMWR, 2010), depending on a variety of biological and environmental factors. Increased larval development time and mortality in *Ae. aegypti* are associated with low temperatures, resulting in decreased transmission (Alto and

Bettinardi, 2013). The same study showed that, if temperatures are lower than 16˚C, larval development is slowed, adult mosquitoes stop feeding, and DENV cannot replicate inside the vector, hence decreasing vector population growth and subsequent transmission.

Higher temperatures shorten the extrinsic incubation period of DENV, increase the development rate of immature mosquitoes, and increase blood feeding rate (Johansson, 2008), hence increasing the likelihood of DENV transmission. Johansson (2008), conducted a 20 year analysis to determine the association between temperature and precipitation with DF in 77 municipalities of Puerto Rico. Monthly variation in temperature was positively associated with DF in most municipalities (Johansson, 2008). However, in areas where temperature and precipitation were already high (30°C and 1,800 mm/month, respectively), increases in environmental variables did not affect the number of DF cases reported (Johansson, 2008).

Dengue in Pan America and the United States

During the 19th century, DEN outbreaks were frequent throughout port cities in the Caribbean, South, Central and North America due to commercial activities (Guzman and Kouri, 2003; Halstead, 2006; Dick et al., 2012). A DEN outbreak in Galveston, Texas in 1918 was followed by another outbreak in 1922 with an estimated 30,000 cases (Rice, 1923). Following the route of the South Pacific Railroad, this DEN outbreak expanded throughout Texas, Louisiana, Florida, and Georgia (Dick et al., 2012). An extended epidemic affecting *ca.* 10% of the population in Florida started in Miami in 1934 and then spread to the rest of Florida and southern Georgia (Griffitts, 1935; Mac Donell, 1935). Between 1941 and 1946, DEN continued to spread throughout the region with multiple outbreaks in Texas (Dick et al., 2012). During 1946-1979, there were no cases of locally acquired DEN in the continental US (MMWR, 2010).

The first case in Texas since 1946 was in 1980 with 14 more cases in Texas in 1986 (Rigau-Perez, Gubler and Vorndam, 1994). From 1980-1992, no further locally acquired cases were reported in the US. On September 1, 2009 a New York state resident whose only recent travel was to Key West, Florida became the first confirmed case of locally acquired DF in the US (MMWR, 2010) since 1986. During the next two weeks, two residents of Key West who had no record of recent travel were confirmed to have DEN. After this, 24 more human cases in local residents were confirmed in Key West (MMWR, 2010). In Florida, there is potential for DENV transmission due to international travelers and visitors to the region, a largely non-immune population, and the invasiveness of both *Ae. aegypti* and *Ae. albopictus*. From 2001 to 2002, 122 patients in the US tested positive for DENV antibodies. Some of the patients were from Hawaii and were the first cases in Hawaii since WWII (Effler et al.,2005; Halstead, 2007).

One of the most important factors in the increasing incidence of DEN is the lack of effective mosquito control (Gubler, 2002; Tolle, 2009; Edillo et al., 2015). Another factor is demographic changes (e.g. high population growth rate and increased urbanization) (Gubler, 1998; Tantawichien, 2012), resulting in poor housing, inadequate water supply/sanitation, thereby increasing oviposition sites due to domestic water-holding containers (Brightmer, 1998; Gubler, 2002; Ligon, 2004; Edillo et al., 2015). Increases in air travel allow DENV (and potentially, its vectors) to spread globally (Gubler and Trent, 1994; Gubler, 1998; Tantawichien, 2012). During the 1980s there was a reduction in public expenditures for mosquito control, thereby causing emergency responses to outbreaks instead of surveillance-based targeted control (Brightmer, 1998). The importance of mosquito control programs (MCPs) are often underestimated because of their inability to create revenue, hence, are often subject to funding cuts (Herring, 2010; Harris et al., 2014; del Rosario et al., 2014). With the emergence and swift

spread of WNV in the US in 1999 resulting in a substantial supplement of government funding, several MCPs were created to combat WNV (Herring, 2010; Vasquez-Prokopec, 2010; Harris et al., 2014). However, WNV has become less of a concern to the public in recent years and MCPs budgets have declined (del Rosario et al., 2014). Mosquito control budget reductions continue today, increasing both the cost of control (e.g. reaction to epidemics) and the number of human cases of vector-borne disease (Herring, 2010; Vazquez-Prokopec et al., 2010). The detection of and response to vector-borne diseases can be severely impaired without a strong surveillance system for human/veterinary cases and potential vectors (Franco et al., 2010; Vasquez-Prokopec et al., 2010). Without CDC funds for vector control and testing, the testing of potential vectors would be halted, causing detection of transmission and new viral introductions into the US to be significantly delayed. This would turn a potentially proactive system into a reactive system, which is currently what the US utilizes (Couzin-Frankel, 2010; Vasquez-Prokopec et al., 2010).

In many countries in Pan America, the lack of water treatment services results in improper water storage and lack of trash collection, causes accumulation of waste receptacles holding rainwater and supports larval development (Whitehorn and Farrar, 2011; Tavodova, 2012). This contributes to urban *Ae. aegypti* infestations (Weaver, 2010; Medeiros, 2011; Simmons, 2012). Global warming also may facilitate DENV transmission due to the geographic expansion of the vectors (Gratz, 199; Weaver, 2010). A rise of 2°C would increase the risk of DENV transmission in South America by five times and new transmission areas would be expected in the southern part of the South American continent (Githeko et al., 2000; Vezzani and Carbajo, 2001).

In 2003, the Integrated Management Strategy for Dengue Prevention and Control (IMS-Dengue) was approved by the Pan-American Health Organization. This six component model includes 1) entomology, 2) epidemiology, 3) environment, 4) healthcare, 5) laboratory, and 6) social communication (Dick, 2012). This strategy utilizes individual modification techniques to decrease risk factors for DENV transmission. The entomology portion establishes a system of entomologic surveillance by applying the Integrated Vector Management approach which focuses on education, environment, public works and the academic sector (Guzman, 2010; Ligon, 2004). Epidemiologic surveillance ensures that public health policies have an interdisciplinary, intersectional and multisectorial focus (Guzman, 2010). Health care ensures that proper training has been administered to health workers to identify treatment and disease properly and inform the community of the warning signs. Pre-travel advice and post-travel follow-up of patients who have traveled to endemic areas is necessary so the patient can have a comprehensive understanding of dengue (Pye, 2012). The laboratory sector ensures that a laboratory is available for the surveillance system which includes an integrated network of available laboratories to call upon if help is needed or an intervention needs to be designed (Guzman, 2010). Finally, social communication strengthens the effectiveness and sustainability of national strategies through community participation.

Dengue and Travel

Dengue is the most diagnosed arboviral illness in travelers (Cleton, 2012). Three to eight percent of travelers with febrile disease are DENV-positive via serology (Cleton, 2012; Mangold and Reynolds, 2013). The majority of DF cases in the US are in US residents returning from vacation in Central and South America (Mangold and Reynolds, 2013). Dengue is underdiagnosed, hence the infection risk could be significantly higher than currently reported

(Cleton, 2012). In a serological survey done in the US between 1986 and 1992, 40% of positive tests came from patients who had travelled to Latin America or the Caribbean (Rigau-Perez, Gubler and Vorndam, 1994). In 2010, 162,058,000 visitors entered the US (WTO, 2012). Brazil is endemic with all four serotypes of DENV and had 5,161,000 visitors in 2010 (WTO, 2012). As travel continues to increase, so does the risk of being bitten by a mosquito infected with DENV (Gubler, 1998; WTO, 2007; Dick, 2012). In 1994, *ca.* 40 million people left the US by air and 50% travelled to tropical countries where DENV is endemic (Gubler, 1998). Many people become infected while traveling but do not exhibit symptoms until after they return home (Gubler, 1998). The constant movement of humans infected with DENV causes repeated introductions of new serotypes into different geographic regions (Rigau-Perez, 1994). By tracking travel to and from DENV-endemic countries, risk assessments may be improved.

Vector Competence for DENV

Mosquitoes acquire pathogens via blood meal (Beerntsen, 2000). Vector competence is the intrinsic permissiveness of a vector to infection, replication and transmission of a virus (Bennett, 2002; Guo, 2013). Estimates of a vector's capacity to transmit pathogens consider cellular, behavioral, environmental, and biochemical factors that influence the association between the vector, the pathogen, and the vertebrate host to which the pathogen is transmitted (Beerntsen, 2000). Vector competence is associated with a number of barriers to infection, i.e. midgut infection and escape, salivary gland infection and escape. In vectors that have a midgut infection barrier, the virus cannot infect and/or replicate in midgut cells (Salazar et al., 2007). The cause of this may be a lack of cell surface receptors for the virus or the midgut cells may not be permissive for infection with the virus (Bennet, 2002). Vectors with a midgut escape barrier may allow the virus to enter and replicate in the midgut, even to high concentrations, but the

virus will not be able to escape midgut tissues. *Aedes aegypti* populations exhibit considerable genetic variation causing a wide range of vector competence, which may explain, in part, the large variance in the epidemiology of DENV (Bennet, 2002). Pathogens and microbes inhabit the same environment prior to infection (e.g. mosquito midgut) and infection is decreased in vectors harboring some species of bacteria (Ramirez, 2012). Bacteria can deter DENV replication by boosting the mosquito's immunity (Ramirez, 2012), whereas fewer bacteria in the midgut decrease barriers to DENV replication (Ramirez, 2012). Midgut bacteria can also increase vector competence for DENV in *Ae. aegypti* (Mourya et al., 2002). Viruses may also change the midgut bacteria composition in *Ae. albopictus* (Zouache et al., 2012). For example, under the condition of the study performed by Zouache et al (2012), infection with chikungunya virus promotes growth of *Protobacteria* and decreases the presence of *Alphaprotobacteria* (Zouache et al., 2012). The geographic expansion of mosquitoes may be related, in part, to the symbiotic relationship between vectors and microorganisms such as bacteria (Zouache, 2012). There are multiple viral replication sites in the mosquito including the midgut, malpighian tubules, thoracic musculature, and salivary glands (Beerntsen, 2000). Mosquito genetics can play a role in the success of pathogen replication (Beerntsen, 2000). However, the genetic makeup of a virus or parasite can also impact whether the virus can replicate in the mosquito (Beerntsen, 2000). Therefore, pathogen-vector interactions are a complicated system and determine whether replication and transmission occur (Beerntsen, 2000). The midgut is a hostile environment for pathogens due to differences in pH, temperature, and blood digestion enzymes (Champagne, 2005). For most pathogens, replicating in and exiting the midgut quickly increases pathogen survival rate (Beerntsen, 2000). Mosquitoes have immune systems and, despite their best efforts, many pathogens are able to develop and replicate within them (Beerntsen, 2000).

Anderson and Rico-Hesse (2006) determined that mosquitoes fed simultaneously on both the American and Southeast Asian DENV-2 genotypes were more likely to become infected with the Southeast Asian (21% of mosquitoes) compared to American (3% of mosquitoes) genotype (Anderson and Rico-Hess, 2006). In the same study, McAllen (Texas) *Ae. aegypti* females were reared at 28˚C with 75% humidity and a 14:10 light/dark cycle and fed defibrinated rabbit blood containing either the Southeast Asian or American strain of DENV-2 virus at 2.5 x 10^8 genome equivalents/mL. After feeding, mosquitoes were kept at 30˚C for up to 14 days (Anderson and Rico-Hess, 2006). In the same study, by day four post infection, 80% of midguts were positive with Southeast Asian genotype compared to 45% infected with the American genotype. The same occurred with the salivary glands where at day seven, 50% of the mosquitoes fed the Southeast Asian strain were virus-positive, while it took until day 14 post-infection for the American strain to reach 50% infection of mosquitoes. It was determined that the Southeast Asian strain replicated to a higher level than did the American strain (Anderson and Rico-Hess, 2006).

The biological makeup of ingested blood may influence vector competence (Beernsten, 2000). Vectors have evolved mechanisms that improve blood uptake from the host and the vertebrate hosts have developed mechanisms to prevent blood loss (Beerntsen, 2000). Antihemostatic mechanisms exhibited by *Ae. albopictus* and *Ae. aegypti* are found in the saliva (Beerntsen, 2000). Components in mosquito saliva are vasodilators, anticoagulants and platelet aggregation inhibitors (Champagne, 2005). Mosquitoes have Adenosine Tri-Phosphate and Adenosine Di-Phosphate (ATP/ADP) diphospholhydrolase. The enzyme works by depleting the ADP and ATP from the bite site so that platelets cannot block the wound and repair cannot take place (Champagne, 2005). The next enzyme is an anticoagulant such as prolixin-S which

facilitates blood flow to the mosquito (Beerntsen, 2000; Champagne, 2005). The ingested blood may display different viscosities due to coagulation in the midgut and this can inhibit ingested pathogens from migrating into and out of the midgut (Beerntsen, 2000). The viscosity of the ingested blood can influence the prevalence and intensity of infection depending on the duration of virions in the midgut (Beerntsen, 2000).

Dengue Vaccines

In order to create a vaccine that will be viable and safe for humans, a life-long tetravalent immunity against all four serotypes of DENV will be needed (Weaver, 2010; Whitehorn and Farrar, 2011). Due to antibodies produced after vaccination, infection with any DENV strain could result in unfavorable outcomes because of activity of non-protective, cross reacting enhanced antibodies (Perng, 2011). An ideal DEN vaccine should have a rapid immunization regime that can fit in with the already established vaccine programs and be administered in one or two doses. It must also be easy to store and transport, affordable, genetically stable, provide long-lasting immunity and should not contribute to immunopathogenesis through vaccine-induced enhancement (Webster, Farrar and Rowland-Jones, 2009). Live attenuated vaccines are at a relatively advanced stage of clinical development (Sabchareon et al., 2002; Durbin, 2011) though the ability to develop a tetravalent live attenuated vaccine against all four DENV serotypes has been difficult. Phase III trials (based on the yellow fever 17D virus) of a live attenuated tetravalent chimeric vaccine produced by Sanofi Pasteur are ongoing (Durbin, 2011). However, the current ideal vaccine model requires three doses given over a 12 month period in order to fully induce the neutralizing antibodies to all four serotypes (Durbin, 2011). DNA vaccines could be another option in the future, which induce humoral and cellular immune responses much like those of live attenuated virus, but do not cause the same safety issues of the

live virus vaccines (Durbin, 2011). In Durbin's (2011) study, only partial protection was observed against DENV-1 while no protection against DENV-2 was observed. There are also recombinant adenovirus vectored DEN vaccines in development that can be administered on a smaller dosing schedule and can induce more potent immune responses than DNA vaccines (Durbin, 2011). Although control for DEN by widespread vaccination has been a priority for the WHO for three decades, a licensed vaccine is still not available (Brandt, 1990; Webster et al., 2009).

Chapter 3 - Dengue in Pan America 2001-2012: Trends in Cases Imported into the United States

**Note: This chapter is formatted as a complete manuscript and will be submitted to a peer-reviewed journal in the future. Hence, there may be some repetition in Chapter 2 (Literature Review) and the Introduction for Chapter 3.*

Abstract

Risk assessments showing the impact of travel on the importation of dengue virus (DENV) are essential to understand the role of human travel in the spread of this mosquito-borne pathogen for which humans are the primary reservoir. Consequently, we calculated the exposure risk of United States (US) citizens traveling into Pan American countries where dengue (DEN) fever is endemic. The number of DEN cases in 51 Pan American countries was compared to the population of the same countries from 2001-2012 as a measure of exposure risk. Travel statistics (i.e. US travelers visiting the 51 Pan American countries) were analyzed and categorized by geographical region (i.e. North America, Central America, Andean, Southern Cone, Hispanic Caribbean and English, French and Dutch Caribbean). Travel patterns for US citizens were compared with subsequent DEN infections for each region visited. We show that US travelers visiting the Dominican Republic exhibited the highest number of imported DEN infections for the period of study. The Pan American country with the most DEN cases in its residents was Brazil (> 1 million reported cases in 2010). The numbers of DEN cases in Pan America continues to rise as does international travel and the geographic range of potential DENV vectors. Hence, in order for DENV risk assessments to improve, we must analyze possible routes of entry for this pathogen. There is also an increased risk of introducing new DENV serotypes into naïve human populations. Regions where all four DENV serotypes are prevalent are at the

highest risk for DHF and DSS. Underreporting and misdiagnosis remains an issue for calculating DENV transmission risk and this is discussed.

Introduction

Dengue fever (DF) is the most diagnosed traveler-related illness with 390-400 million cases/year worldwide (Bhatt, 2013; Cross, 2013; Yacoub and Wills, 2014) and an incidence rate of *ca.* 2.5-5% of the > 2 billion people at risk (Suaya, Shepard and Beatty, 2006) (Figure 1). Only an estimated 3-8% of symptomatic travelers are positive for DENV via serological tests (Cleton, 2012). Infection with one DENV serotype may cause a range of symptoms (e.g. asymptomatic, flu-like). However, a simultaneous and/or sequential infection with different serotypes increases the risk of serious illnesses such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which could lead to death (Durbin, 2011). Often, patients are unaware of initial infection and experience severe symptoms when superinfection with another serotype occurs (Durbin, 2011). An understanding of human travel patterns between DEN-endemic countries and the US will improve risk assessments and identify potential routes of entry for DENV. The geographic ranges of the four DENV serotypes are expected to expand with international travel as humans are the primary reservoirs (Gubler, 2012). This increases the likelihood of multi-serotype epidemics that could impact public health. Risk assessments showing the impact of travel on importation of DENV are essential to understand the role of human travel in pathogen spread.

Pan America can be categorized into four regions, i.e. North America, Central America, South America, and Caribbean. Most DEN cases among US citizens occur as a result of endemic transmission in Puerto Rico, a US territory (CDC, 2013). In 2010, 162,058,000 visitors entered

the US (WTO, 2011). Of those, 1,197,866 were Brazilian (OTTI, 2010). Dengue is endemic in Brazil where all four serotypes of DENV circulate (PAHO, 2009). Travelers returning to the US from Brazil accounted for 70% of DEN cases between 1998 and 2008 (Mangold and Reynolds, 2013, San Martin et al., 2010).

Globally, > 2 billion people/year are at risk for DEN infection and > 21,000 DEN-related deaths/year occur, yet the range of expected symptoms makes it difficult for medical diagnoses without a serological test (Simmons, 2012). Studies have identified young age, high body mass index, female sex, virus serotypes, and virus genotype as risk factors for severe DEN (Simmons, 2012). Patients recovering from DHF may experience symptoms such as fatigue for up to six months (Simmons, 2012). These increased risks are important due to the economic burden of lost work time and associated medical costs (MMRW, 2010; Rothman, 2013, Gubler 1998). A study of 2012 healthcare costs in the Philippines reported $345 million ($3.26 per capita) spent in direct medical costs for DEN patients (Edillo et al. 2015). In Pan America, DEN cost *ca.* $2.1 billion/year in medical costs (Shepard, 2011). In the US, each person hospitalized from DEN pays *ca.* $17,803 and less severe cases cost *ca*. $1,610 (Shephard, 2011). The median cost of treatment throughout Pan America is $1,227 USD (Shephard, 2011). Costs vary substantially between countries due to the value of the dollar and the variance of direct cost, i.e. difference in costs between hospitals (Gonzalez, 2011). Proper health care facilities are not accessible to all DEN patients, hence underreporting occurs (Dick, 2012)

Dengue emerged in Africa between the 15th and 19th centuries during slave trades (Weaver, 2010). Dengue virus was first isolated in 1943 and serological tests were subsequently made available (Dick, 2012). Currently, DEN cases are diagnosed based on symptoms rather

than serological tests that are used simply to confirm infection for research (Dick, 2012). Before 1970, DEN had only been detected in nine countries; however, by 1996, 102 countries had experienced epidemics (Dorji, 2009). In 1962, a comprehensive mosquito control effort was developed and implemented by the Brazilian government, Pan American Health Organization (PAHO) and the Rockefeller Foundation (Dick, 2012). Although this effort attempted to eradicate the primary DENV vector, *Aedes aegypti* L., re-infestation occurred when plans deteriorated due to the loss of political interest (Dick, 2012, Mangold and Reynolds, 2013). Insufficient community participation and a lack of support from the health sector added to the deterioration of the eradication program (Dick, 2012). By 1980, DEN outbreaks increased globally and, in 1981, Cuba experienced an outbreak with 344,203 cases, including > 10,000 DHF cases and > 150 deaths (Dick, 2012). From 2000-2012, all four DENV serotypes were found in Pan America, causing the highest number of cases to date (Dick, 2012).

Aedes aegypti and *Ae. albopictus* Skuse are the two primary vectors of DENV and are distributed through Pan America (Simmons, 2012). *Aedes albopictus*, a day-biting species originally found in Asia, began geographic expansion in the 1980s and is still expanding today (Lambrechts, 2010). This mosquito species was introduced into the US in 1985 from Asia in a shipment of tires (Hawley et al., 1987; Gubler, 1998; Benedict et al., 2007) and is anthropophilic (Higa, 2011). *Aedes aegypti* takes multiple blood meals, hence, this species may infect multiple humans during a single gonotrophic cycle (Weaver, 2010). This mosquito species will stop blood feeding when disturbed and either return to the same host or a different host to complete a blood meal (MMWR, 2010; Gubler, 1998). While both species oviposit in artificial containers with standing water, *Ae. albopictus* often oviposits in natural containers (Lambrechts, 2010). *Aedes aegypti* is considered a better DENV vector because of its adaptation to urban environments in

tropical cities in close proximity to humans. *Aedes albopictus* is better adapted to suburban and rural settings with vegetated areas facilitating its preferred larval development and resting sites (Lambrechts, 2010). Warm temperatures in many Pan American countries shorten the extrinsic incubation period of DENV and increase the development rate of the vector. These factors increase the likelihood of a vector successfully transmitting a virus (Johannson, 2008). Rapid expansion of international and domestic human travel, urban sprawl, and insufficient vector control may facilitate the geographic expansion of DENV (Dick, 2012).

Some of the most visited countries in the world are in Pan America (CTS, 2011). Here, we conduct a risk assessment for 2001-2012 based on: 1) visitors from 51 Pan American countries traveling to the US, and 2) the total number of residents and DEN cases in 51 Pan American countries. We expect this analysis to facilitate models interested in assessing risk of traveler-imported infections and the risk of importing DENV into the US.

Materials and Methods

Travel statistics for 51 Pan American countries were tabulated from the Compendium of Tourism Statistics and the Office of Travel and Tourism Industries between 2001-2012 (Table 1) (OTTI, 2013). Countries were categorized by geographical region (i.e. North America; Central America; South America and Caribbean) (US Census Bureau, 2013). Populations of these 51 countries were tabulated (Table 2) (US Census Bureau 2013). Numbers of clinical DEN cases were identified and counted from the Pan American Health Organization (PAHO, 2013) and the Centers for Disease Control and Prevention (Jennifer Lehman [CDC], personal communication). Numbers of DEN cases were compared to annual travel statistics and populations for each region. Regions with the most visitors to the US were ranked and analyses were conducted for 18

countries whose residents visited the US most frequently. The *incidence rate* was determined (the number of DEN cases in the country/the population of the country). Next, the *infection rate* was calculated (the number of DEN cases reported for each country was divided by its respective population and multiplied by 100,000). To determine the number of infected people potentially traveling to the US, we multiplied the infection rate by the number of individuals traveling to the US from these 18 countries for each year between 2001-2012. To determine the risk of traveling to an endemic country and becoming infected, the number of US citizens traveling to each region was multiplied by the infection rate for that region. This method was repeated for each consecutive year studied. The CDC provided information on DEN cases imported into the US by US citizen travelers from 2003-2011 (Jennifer Lehman, personal communication). The numbers of DEN cases from each of the 18 Pan American countries was divided by the total number of cases for each year to determine the visited country where the highest rate of infections occurred in US travelers.

Maps were created using Environmental Systems Research Institute (ESRI) ArcMap 10.1 (Redlands, California) and 18 Pan American countries were selected as map layers. Infection rates (PAHO, 2001-2012) calculated were input into attribute tables for specific countries. Graduated colors were used to display the numbers of DEN infections/100,000 people for each country. Maps were created for years 2004, 2008 and 2012 because available data was more complete starting in 2004 for the countries relevant to our study.

Results

We generally observed yearly increases in international travel to the US from all regions of Pan America from 2001-2012 (Figure 2). For the time period studied, most Pan American

DEN cases occurred in Brazil (South American Region) in 2010 (> 1 million reported cases) (Table 1). For US citizens, the highest number of imported DEN cases was observed in continental US travelers visiting the Dominican Republic, closely followed by Puerto Rico (Caribbean Region) (Jennifer Lehman, CDC, personal communication). Table 1 shows that reported clinical cases of DEN have increased where surveillance systems have become a priority, such as Brazil (mandatory reporting started in 2007). Until 2009, DEN was not a nationally reportable disease in many Pan American countries, including the US; hence, cases prior to 2009 may be underreported. Some countries only report serologically positive cases, hence, physician-diagnosed cases (relying solely on symptoms) may be underreported (PAHO, 2010). The South American Region has the highest number of DEN cases over the 12 years studied (Figure 3). Infection rates in all regions have increased since 2001 with the worst epidemics occurring in 2010 (Figure 4). Of the 18 countries studied, Brazil (3rd highest number of travelers to US) had the highest number of DEN cases. The DEN infection rate was highest in the South American region (341 cases/100,000 people in 2010), primarily attributed to Brazil. The Caribbean and South American regions both experienced DEN epidemics in 2010 while Central America had an epidemic in 2009 (Figure 3). However, there is a large population difference between the South American Region and other regions such as the Caribbean and Figure 4 accounts for these differences, i.e. regional infection rate with the population taken into account (incidence rate). While South America still has the highest infection rate, including the population in the equation demonstrates the severity of DEN in the Caribbean.

We determined the potential number of infected travelers entering the US for each year studied (Figure 5). In 2010, Brazil documented four DENV serotypes and experienced the largest DEN outbreak in Pan America during the period of study (2001-2012). Mexico had the highest

number of potentially infected travelers visiting the US, i.e. highest in 2009 with > 29,000 potentially infected travelers (Figure 6). Figures 7-9 show DEN incidence (serologically positive cases/100,000 people) in 2004, 2008 and 2012, respectively. In 2004, several countries had incomplete data available, due, in part, to underreporting. Countries with higher infection rates have at least three DENV serotypes circulating (e.g. Brazil, Venezuela, and Mexico) and its residents travel to the US most frequently. Table 2 shows that the number of DENV serotypes in most countries increased between 2004 and 2012.

Discussion

Countries endemic for DEN pose a higher risk for travelers and thus create a higher risk for spread of DENV back to the home country. As travel and geographic range of potential DENV vectors continue to increase, incidence of DEN will likely increase (Gubler, 1998). There is also an increased risk of introducing new DENV serotypes into naïve populations. Highly traveled regions where all four DENV serotypes are prevalent (e.g. South America) pose the greatest risk. While underreporting and misdiagnosis remain an issue for calculating DENV transmission risk, we observed increases in case frequency. Because DEN is not nationally reportable in many countries, it is often underreported. Many patients infected with one serotype of DENV are asymptomatic or experience flu-like symptoms and do not seek medical treatment (Gubler, 2004). Others cannot afford to go to the doctor or do not have easy access for treatment and, therefore, go unreported (Gubler, 1998). There is a lack of uniform application of the case definition of DEN and some countries have instituted their own case definitions (Deen, et al., 2006). In addition, complicated reporting systems and/or lengthy reporting requirements may reduce motivation of health-care workers to submit positive test results (Suaya et al., 2006). Underreporting impacts public health because it is an enormous barrier to obtaining an accurate

risk assessment. In Belo Horizonte, Brazil, the level of reporting of hospitalized DEN patients was *ca.* 63% between 1997 and 2002 (Duarte and Franca, 2006). Tests for virus by cell culture or nucleic acid detection (PCR) require sophisticated laboratories (Pediatric Dengue Vaccine Initiative [PDVI], 2006) and there is limited accuracy in rapid tests (Suaya et al., 2006). As a result, the mobilization of resources from the local, national and international communities for the elimination of the vector and better infection care (Suaya et al., 2006) needs improvement.

Aedes albopictus and *Ae. aegypti* are both found in the US, hence traveler-imported cases are a concern for some regions since local mosquito populations may become involved in subsequent transmission. Florida had 125 locally transmitted cases from 2009-2014, while Hawaii (4 cases in 2011), Texas (24 cases from 2013) and New York (1 case in 2013) also experienced locally transmitted cases (MMWR, 2009, 2010; USGS, 2014). There were 177 human DEN cases from 22 different states reported to CDC Arbonet in 2009, 642 cases from 39 states in 2010, 245 cases from 32 states in 2011, 544 cases from 34 states in 2012, 772 cases from 41 states in 2013, and 357 cases from 37 different states in 2014 (Jennifer Lehman, personal communication; USGS, 2014).

There is currently no DEN vaccine (Meltzer and Schwartz, 2009) and there is great concern for people who have already been infected with one or more serotypes and how they would react to the vaccine (Messer et al., 2014). As more serotypes become endemic in more countries, the risk for becoming infected and the potential for DHF increases. With > 43 million travellers entering the US by air from Pan American countries, the risk for travel related DEN exists. Continued surveillance and risk assessment is needed to prevent further DEN expansion and reduce the risk of importation.

Chapter 4 - Effects of Blood Meal Source on *Aedes albopictus* Life Table Characteristics and Vector Competence for Dengue Virus

**Note: This chapter is formatted as a complete manuscript and will be submitted to a peer-reviewed journal in the future. Hence, there may be some repetition in Chapter 2 (Literature Review) and the Introduction for Chapter 4.*

Abstract

Commercially available blood can be used as an alternative to live animals to maintain mosquito colonies and deliver infectious blood meals during research studies. However, the extent to which artificially delivered blood sources affect mosquito life table characteristics of *Aedes albopictus* and vector competence for dengue virus (DENV) is unknown. Consequently, we analyzed the extent to which two blood sources affected life table characteristics (i.e. fecundity, fertility, hatch rate, adult survival) and vector competence (infection, dissemination, transmission) of *Ae. albopictus* for DENV. Two types of blood (N = 40 mosquitoes/group) were tested at two extrinsic incubation temperatures for DENV-infected and –uninfected mosquitoes as follows: 1) defibrinated, 27°C; 2) citrate, 27°C; 3) defibrinated, 30°C; 4) citrate, 30°C. Fully engorged mosquitoes were transferred to individual cages containing an oviposition cup and substrate. The presence of eggs was observed daily and, if eggs were observed, the substrate was removed and the number of eggs was counted (fecundity) for each female. Eggs were allowed to hatch and larvae were counted (fertility) for each female. At 14 and 21 days post feeding, 15 mosquitoes were taken from each group and tested for DENV in bodies (infection), legs (dissemination), and saliva (transmission). Mosquitoes fed DENV-infected defibrinated blood showed significantly higher DENV body titer ($P = 0.034$) and fecundity ($P = 0.032$), as well as faster hatch time ($P = 0.039$) compared to mosquitoes fed DENV-infected citrated blood. Temperatures tested here did not ($P > 0.05$) affect any factor measured. No differences were

observed in DENV leg titers between treatments. DENV transmission was observed in all groups 14 days post infection and was observed in all but the 30°C defibrinated blood group at the 21 day post infection time point. Infected mosquitoes showed higher fecundity than uninfected mosquitoes ($P = 0.001$); however, fertility was lower in infected compared to uninfected mosquitoes ($P = 0.001$). Eggs of DENV-infected mosquitoes hatched faster than the uninfected groups ($P = 0.005$). We expect the findings of this study to improve methods for mosquito colony propagation and inform research using artificial blood delivery methods to assess vector competence.

Introduction

Aedes albopictus Skuse is an anthropophilic mosquito that was first detected in the United States (US) in Houston, Texas in 1985 in a shipment of tires from Asia (Hawley et al., 1987; Moore and Mitchell, 1997; Gubler, 2002; Alto and Bettinardi, 2013) and has since spread to > 678 counties in *ca.* 25 states (Hawley et al., 1987; Moore and Mitchell, 1997; CDC, 2014). *Aedes albopictus* is the most invasive mosquito species in the world and has significant public health importance due to its ability to transmit multiple pathogens including West Nile virus, La Crosse virus and dengue virus (DENV, family *Flaviviridae*, genus *Flavivirus*) (Benedict et al., 2007).

In the US, each person hospitalized from DEN pays approximately $17,803 and less severe cases cost *ca*. $1,610 (Shepard, 2011). The median cost of treatment throughout Pan America is $1,227 (Shephard, 2011). A study reports the economic cost of DEN cases in the Philippines in 2012 was $345 million ($3.26 per capita), a 54% increase from 2010 (Edillo et al., 2015). For laboratories studying DEN and other infectious diseases, mosquito colonies are maintained with blood (either live animals or artificial delivery systems) (Clements, 1992). One

environmental factor that affects biological processes of mosquitoes (including their interaction with viruses and survival) is temperature (Halsted, 2008; Alto and Bettinardi, 2013; Brady et al., 2013; Carrington et al., 2013). Survival of the vector is a key component of transmission of a vector-borne pathogen (Reisen et al., 1980). Increased survival time allows the vector more opportunities to produce offspring, disperse, and, transmit pathogens in subsequent blood feedings (Brady et al., 2013; Parham and Michael, 2010; Barbazan et al., 2010). Under laboratory conditions, the effects of temperature can be quantified and other causes of mortality found in the field such as predation or disease are removed (Brady et al., 2013). Laboratory conditions are not expected to produce the same effects on life table characteristics as field conditions (Lambrechts et al., 2011; Brady et al., 2013), but are often used as a proxy for understanding aspects of field dynamics. Vector competence includes the mosquito's susceptibility to infection and the capacity to transmit a pathogen to a host after ingestion of an infectious blood meal (Kramer and Ebel, 2003; Salazar et al., 2007; Carrington et al., 2013). A previous study of *Ae. aegypti* describes how females exposed to a large diurnal temperature range *ca.* 20°C mean showed a higher rate of DENV dissemination after 28 days compared to females reared under a constant controlled temperature (dissemination rates of 100% vs. 42%, respectively) (Carrington et al., 2013). Increases in extrinsic incubation temperatures have been associated with enhanced vector competence in several vector-virus systems (e.g. Richards et al., 2007; Richards et al., 2009; Kilpatrick et al., 2008).

For many laboratories, commercially available blood is used as an alternative to live animals when feeding mosquitoes. It replaces the time required to maintain live animals which are expensive to keep and subject to government regulation (Turell, 1988). However, laboratories that employ artificial blood delivery systems must also consider the effect of the

blood source on the life table characteristics (and possibly vector competence) of the mosquitoes (Richards et al., 2012). This must also be considered when comparing results of experiments where different types of blood are used. Blood can be treated with anticlotting additives such as sodium citrate and/or go through defibrination (e.g. fibrin separated from it during the clotting process) to prevent coagulation. Experimental results may be influenced if there are physiological changes that manifested in the mosquitoes from using different blood sources (Richards et al., 2012).

Previous research has assessed the effects of blood meal source on life table characteristics of *Culex pipiens quinquefasciatus* (Richards et al., 2012), *Culex theileri* (Demirci et al., 2014), *Ae. aegypti* (Bennett, 1970; Watts et al., 1987) and five *Anopheles* species (Phasomkusolsil, 2013). Previous studies have also looked at the effects of extrinsic incubation temperature (EIT) on vector competence in *Culex nigripalpus* for Saint Louis encephalitis virus (Richards et al., 2012), *Culex pipiens quinquefasciatus* for Saint Louis encephalitis virus (Richards et al., 2009), West Nile virus (Richards et al., 2007) and on *Ae. albopictus* (Richards et al., 2012) and *Ae. aegypti* for DENV (Watts et al., 1987). The aim of the current study is to improve mosquito colony propagation techniques and to inform future research using artificial blood delivery methods and different EITs to assess vector competence and life table characteristics of *Ae. albopictus*. No studies have assessed the effects of blood source on life table and vector competence characteristics for *Ae. albopictus* infected with DENV.

Materials and Methods

Mosquitoes and Virus

Ae. albopictus eggs (F_8) originating from New Orleans, Louisiana were reared at 27°C under standard conditions to produce similar sized mosquitoes (Richards et al. 2009) with 70-

80% relative humidity and a 14:10 (light:dark) cycle. Eggs on seed germination paper were placed in 16 plastic pans (34.3 cm x 25.4 cm) (Bioquip, Rancho Dominguez, CA) with approximately 1200 mL of tap water/pan. Larvae were fed a 2:1 mixture of Brewer's yeast and liver powder each day. Pupae were transferred to 200 mL plastic cups containing approximately 150 mL of tap water, placed into square cages (30.5 cm height x 30.5 cm width x 30.5 cm depth) (Bioquip, Rancho Dominguez, CA), and provided 20% sucrose *ad libitum.* Plastic cups containing pupae were removed after three days to limit the age difference of adults. A Southeast Asian DENV-2 (16803 strain) was passaged once in the mosquito *Toxorhynchites amboinensis* Doleschal, twelve times in Vero cells and twice in C6/36 cells.

Blood Meal Preparation and Infection Study

A T-75 flask containing a monolayer of African green monkey kidney (Vero) cells was inoculated with 0.15 mL DENV. Next, 12 mL of Medium 199 with Earle's salts (M199E), penicillin/streptomycin, fungizone, and fetal bovine serum (FBS) was added to the flask and incubated at 35°C with 5% CO_2 for six days. A 1:1 ratio of DENV supernatant and either citrated or defibrinated bovine blood (warmed to 35°C for 10 minutes) was mixed and blood soaked cotton balls were used to deliver blood meals to mosquitoes. Mosquitoes in the uninfected groups received a 1:1 ratio of M199E:uninfected citrated or defibrinated blood.

Twenty-four hours prior to blood feeding, sucrose was removed from mosquito cages and replaced with water in order to encourage blood feeding. Figure 10 shows the study design where *ca.* 1,000 female *Ae. albopictus* were used to account for an expected 35% feeding rate. Mosquitoes were allowed to feed for 45 minutes and categorized in the following groups: 1) DENV-infected defibrinated blood, EIT 27°C (ID27), 2) DENV-infected defibrinated blood, EIT 30°C (ID30), 3) DENV-infected citrated blood, EIT 27°C (IC27), 4) DENV-infected citrated

blood, EIT 30°C (IC30), 5) uninfected defibrinated blood, EIT 27°C (UD27), 6) uninfected defibrinated, EIT 30°C (UD30), 7) uninfected citrated blood, EIT 27°C (UC27), 8) uninfected citrated blood, EIT 30°C (UC30) (Figure 10). We observed *ca.* 37% feeding rate in all groups, with no difference between citrated and defibrinated groups. We collected 0.1 mL of each blood meal (placed in 2 mL microcentrifuge tubes with 0.9 mL of M199E media) for later analysis to determine blood meal DENV titer. Five freshly fed mosquitoes/group were collected to determine the amount of virus imbibed by mosquitoes. All samples were stored at -70°C until subsequent testing. After blood feeding, mosquitoes were immobilized with cold and fully engorged specimens were transferred individually to separate 0.5 L cardboard cages containing a black oviposition cup (60 mL) glued to the bottom of the cage, a second (clear) oviposition cup placed inside the black oviposition cup, and a 2.5 cm x 2.5 cm oviposition substrate (seed germination paper) clipped to the side of the clear cup. The second clear cup was used to facilitate removal and counting of the eggs during later processing (unpublished data). Partially fed and unfed mosquitoes were discarded. Mosquitoes were provided 20% sugar *ad libitum*. Mosquito survival was monitored daily for the duration of the experiment. As mosquitoes in the infected group died, they were placed (within 24 h of death) in individual 2 mL microcentrifuge tubes with 0.9 mL of M199E media and 2 stainless steel BBs and stored at -70°C until subsequent testing for virus titer.

Three days post-blood feeding, 25 mL of tap water was added to each ovicup so that the oviposition substrate was moistened. The presence of eggs was assessed daily by visual inspection. Two days after eggs were observed, the corresponding mosquito was aspirated from the container, the clear oviposition cup was removed, and the eggs on the oviposition strip and floating in the cup were counted (fecundity) using a dissecting microscope. Egg strips were

submerged in the water contained in the respective oviposition cup, along with larval food administered *ad libitum*. Oviposition cups were returned to incubators and eggs allowed to hatch. The date eggs hatched was recorded for each cage. Approximately seven days post-hatching, all larvae were counted (fertility) using a dissecting microscope. Larvae hatching from eggs of adult females from the uninfected group were counted and sacrificed. Larvae hatching from eggs of DENV-infected adult females were counted and allowed to reach adulthood. Adult females and males from each brood in the DENV-infected group were pooled by cage (brood of a single female) and frozen (-70°C) for future testing of transovarial DENV transmission.

At 14 and 21 days post blood feeding, 15 mosquitoes were aspirated from each infected treatment group. Mosquitoes were chilled, wings removed, and legs transferred to micro-centrifuge tubes containing 0.9 mL M199E, penicillin/streptomycin, fungizone, FBS, with two stainless steel BBs. Live mosquito bodies were secured to a glass surface and separate capillary tubes containing *ca.* 20 uL FBS were placed over each proboscis following standard methods (Anderson et al., 2010; Richards et al., 2012). After mosquitoes were allowed to salivate for 45 minutes, capillary tubes were removed and the contents were expelled into separate tubes containing 0.9 mL M199E media with two BBs. Then the bodies were placed in a third corresponding tube so that each mosquito had three tubes (infection [body], dissemination [legs], transmission [saliva]).

Processing Samples

Samples were processed using previously established methods (Richards et al., 2007). Mosquito samples were homogenized using a TissueLyser II (Qiagen, Valencia, California) for three minutes at 25 Hz. Samples were then centrifuged (4°C) at 4000 RPM for four minutes. Nucleic acids were isolated from 0.14 mL of samples using the QIAamp Viral RNA Mini Kit

(Qiagen, Valencia, California) following the manufacturer's protocol. The amount of viral RNA (calculated in plaque forming unit equivalents; PFUeq) in each sample was determined using TaqMan quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) with the LightCycler® 480 Instrument (Roche, Basel, Switzerland) and the Superscript III One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA) using a program previously established by Richards et al. (2007). Samples were amplified as follows - DENV-2 samples: 48ºC for 30 min, 95ºC for 2 min, 45 cycles at 95ºC for 15 s and 60ºC for 30 s, and finally 40ºC for 30 s. Dengue virus primer and probe (FAM dye with black hole quencher) sequences used to identify the capsid genomic region are as follows: Forward primer: CAT GGC CCT KGT GGC G (237-251); reverse primer: CCC CAT CTY TTC AGT ATC CCT G (305-284); probe: TCC TTC GTT TCC TAA CAA TCC (254-274) (Callahan et al. 2001).

Statistical Analyses

Variables analyzed are listed in Table 3. Analysis of variance (ANOVA; $P < 0.05$) (IBM SPSS version 20, Armonk, New York) was used to determine differences in rates of 1) fecundity, 2) fertility, 3) oviposition rate, and 4) hatch time between different types of blood (citrated or defibrinated) and EITs (27°C or 30°C) (Table 4). We further analyzed (ANOVA) differences in these four variables between mosquitoes fed uninfected or infected blood (Table 5). The differences in, 1) infection rate, 2) dissemination rate, 3) horizontal transmission rate 4) transovarial transmission rate, 5) body titer, 6) leg titer, and 7) saliva titer were analyzed using ANOVA for mosquitoes at 14 and 21 days post blood feeding (Table 6). The effects of blood meal type and status of infection on the lifespan of adult females was ascertained using non-parametric survival analysis and Kaplan Meier tests ($P < 0.05$).

Results

Blood Meal Titer

Blood meals containing infected defibrinated bovine blood showed 7.4 ± 0.3 $\log_{10}$ PFUe DENV/mL and infected citrated bovine blood showed 7.3 ± 0.1 $\log_{10}$ PFUe DENV/mL. No significant difference was observed in blood meal titers between types of blood (F = 0.092, P =0.795, df =1,1).

Effects of Blood Source and EIT on Life Table Characteristics

Mosquitoes fed uninfected citrated bovine blood had significantly higher fecundity compared to those fed uninfected defibrinated bovine blood (Table 4, Figure 11). Fertility was also significantly higher in the uninfected citrated group than in the uninfected defibrinated group (Table 4, Figure 12). Mosquitoes fed uninfected blood (citrated or defibrinated) and incubated at 30°C had a significantly faster hatch time compared to mosquitoes fed uninfected blood and incubated at 27°C, but no differences were observed in fecundity, fertility or oviposition time between blood types and EITs (Table 4, Figures 11-14). Blood meal source (regardless of EIT) did not affect oviposition time or hatch time in mosquitoes fed uninfected blood (Figures 13 and 14). Mosquitoes in the uninfected group fed different sources of blood and incubated at different EITs (blood source * EIT) showed no significant differences in life table characteristics (Table 4).

Mosquitoes in the DENV-infected group fed defibrinated blood exhibited significantly faster oviposition time than DENV-infected mosquitoes fed citrated blood, but showed no significant differences in fecundity, fertility or hatch time between different blood sources or EITs (Table 4, Figures 11-14). Effects of blood source on life table characteristics in DENV-infected mosquitoes did not change with EIT (blood source * EIT) (Table 4).

Effects of Blood Source and EIT on Vector Competence

Infection rate was 100% for mosquitoes sampled at both 14 and 21 days post-blood feeding (Figures 15 and 16). No significant differences were found in dissemination rates between blood type and EIT for either the 14 or 21 day incubation period (Table 6; Figures 15 and 16). At the 21 day incubation period (but not for the 14 day incubation period), horizontal transmission rate was significantly higher in mosquitoes fed citrated blood (Table 6); however, mosquitoes in the 21 day group fed defibrinated blood and incubated at 30°C did not show any horizontal transmission. Consequently, statistical tests could not be conducted for the interaction effects between blood source and EIT on horizontal transmission. Vertical (transovarial) transmission was not found in any group.

Effects of Infection Status on Life Table Characteristics

At 14 days post infection (dpi), body DENV titers were significantly higher in mosquitoes fed defibrinated blood (7.4 ± 0.09 PFUe DENV/mL) compared to citrated blood (7.0 ± 0.05 PFUe DENV/mL) (Table 6, Figure 15). At 14 dpi, no significant differences were observed in leg titers between mosquitoes fed citrated or defibrinated blood, but saliva titers were significantly higher in the citrated group (Table 6, Figure 15). At 21 dpi, body, leg and saliva titers were not affected by blood source, EIT or blood source * EIT (Table 6; Figure 16). Significantly higher body ($P = 0.000$), leg ($P = 0.000$) and saliva ($P = 0.000$) titers were observed at the 14 day compared to the 21 day time point (Figures 17-18).

Fecundity was significantly higher in mosquitoes in the infected group compared to uninfected mosquitoes (Figure 11, Table 4). Blood source and infection status significantly increased fecundity (Figure 11, Table 6). Fertility was not significantly affected by blood source

(Figure 12, Table 4). Oviposition time was significantly faster in the citrated group among both infected and uninfected mosquitoes (Figure 13, Table 4). Hatch time was significantly faster in mosquitoes in the infected group compared to the uninfected group (Figure 14, Table 4).

Mosquito Survival

At both 27°C and 30°C, mosquito survival was significantly lower in the infected group ($P = 0.046$ and $P = 0.011$, respectively) compared to the uninfected group (Figures 19 and 20, Table 7). Blood source and EIT did not affect survival among infected or uninfected groups (Figures 19 and 20). In the 27°C group, the uninfected groups experienced ≥ 80% survival. However, both infected citrate and infected defibrinated groups experienced a dramatic drop in survival after two days post blood meal and this decline in survival levelled off at eight days post blood meal. In the 30°C group the infected citrated group experienced the lowest survival rate with only 40% surviving to 21 days post blood meal, while the infected defibrinated group experienced 81% survival, similar to the uninfected citrate group at 83%. The 30°C uninfected defibrinated group experienced 62% survival. As with the 27°C group (not including the infected citrated group which levelled off after day 14) the survival rate decreased after 2 days post blood meal and levelled off after seven days post blood meal. To account for the skewed curve that would appear if mosquitoes purposefully taken at 14 dpi were present, we excluded them from the survival curve.

Discussion

The interactions between vectors and viruses are complex and can be affected by both environmental and biological factors. In the current study, defibrinated bovine blood and citrated bovine blood impacted *Ae. albopictus* life table characteristics (i.e. fecundity, fertility, oviposition time, hatch time, infection rate, dissemination rate, and horizontal transmission rate)

with mosquitoes fed citrated blood exhibiting higher fertility and fecundity. The higher EIT tested here (30°C) was associated with increased (+ 11%) fertility in groups fed citrated blood and decreased hatch time in the uninfected group, but did not affect fecundity or oviposition time in any group. Temperatures tested here did not affect adult survivorship in any group. We show that EITs of 27°C and 30°C did not affect infection or dissemination rates in *Ae. albopictus* fed either DENV-infected defibrinated or citrated blood, but did affect horizontal transmission. Horizontal transmission was significantly lower in the 30°C group, regardless of blood source. Alto and Bettinardi (2013) evaluated *Ae. albopictus* fed defibrinated bovine blood containing 7.1 ± 0.14 $\log_{10}$ PFU DENV/ml and incubated at 20°C, 25°C, or 30°C. The same study showed that EIT did not affect *Ae. albopictus* susceptibility to infection with DENV at 14 and 21 days post blood meal at 25°C or 30°C. However, a significant difference in dissemination was observed in all groups at 25°C compared to the 30°C treatment group, although the aforementioned study did not test transmission. Vertical (transovarial) transmission was not observed in our study, but has been well documented in the past under varying conditions (Rosen, 1988; Lee et al., 1997; Joshi et al., 2002; Lee and Rohani, 2005; Buckner et al., 2013). The lack of transovarial transmission we observed could be due to low mosquito body titers at the time of oviposition.

In a previous study using *Ae. aegypti* fed DENV blood meals and incubated at a standard temperature of 26°C with daily diurnal temperature fluctuations of 0°C, 10°C and 20°C, DENV dissemination and transmission occurred within a week or less (Lambrechts et al., 2011). In another study performed using Ae. *aegypti* housed at (26°C, 28°C and 30°C) and fed blood meals containing DENV- 2 and -4, the extrinsic incubation period (EIP) was decreased from nine days to five days between the 26°C group to the 30°C group (Rohani et al., 2004). Temperatures ≥ 35°C, are detrimental to mosquitoes, causing inhibited development, increased mortality, and

impairment of reproductive function (Rohani et al., 2004; Carrington et al., 2013; Carrington et al., 2013). Up to a point, higher EITs facilitate DENV transmission because the virus propagates and disseminates faster within the vector (Carrington et al., 2013). Anderson and Rico-Hesse (2006) determined that mosquitoes fed simultaneously on both the American and Southeast Asian DENV-2 genotypes were more likely to become infected with the Southeast Asian (21% of mosquitoes) compared to American (3% of mosquitoes) genotype (Anderson and Rico-Hesse, 2006). McAllen (Texas) *Ae. aegypti* females were reared at 28˚C with 75% humidity and a 14:10 light/dark cycle (Anderson, 2006). The blood meal contained defibrinated rabbit blood and either the Southeast Asian or American strain of DENV-2 virus at 2.5 x 10^8 genome equivalents/mL. After feeding, mosquitoes were incubated at 30˚C for up to 14 days (Anderson and Rico-Hesse, 2006). In the same study, by day four post infection, 80% of midguts were positive with Southeast Asian genotype compared to 45% infected with the American genotype. The same occurred with the salivary glands where at day seven post blood feeding, 50% of the mosquitoes fed the Southeast Asian strain were virus-positive, while it took until day 14 post-infection for the American strain to reach 50% infection. It was determined that the Southeast Asian strain replicated to a higher level than did the American strain (Anderson, 2006).

The time required for the EIP of DENV is eight to 14 days (Axford et al., 2011; Miranda and Johansson, 2012; Goncalves et al., 2014). Our findings that mosquitoes at 21 days post blood feeding had significantly lower body, leg, and saliva titers compared to 14 day time point concur with Salazar et al. (2007) using *Aedes aegypti* mosquitoes. The same study showed that viral titers decreased significantly at 14 and 21 days compared to seven days post infection (Salazar et al., 2007). Several studies look at earlier points in EIP [e.g. Chan and Johansson (2012); Alto et al. (2014)] and have shown that viral titers were highest 7-10 days post infection.

We found significant differences in fertility and fecundity in uninfected groups fed different sources of blood with mosquitoes feeding on citrated blood laying *ca.* 11 more eggs per female (fecundity) and fertility increasing by 14% compared to uninfected mosquitoes feeding on defibrinated blood. Significant differences in oviposition time and hatch time were also found in the infected groups. Oviposition time was faster and decreased by 1.2 days in mosquitoes fed infected defibrinated blood compared to infected citrated blood. Hatch time was also faster and decreased by 0.9 days in the infected group compared to the uninfected group. Body titers were also significantly higher in mosquitoes fed infected defibrinated compared to citrated blood, but leg and saliva titers were not significantly affected by blood source. Host blood sources affect the duration of the gonotrophic cycle and fecundity in *Ae. triseriatus* (Mather and DeFoliart, 1983). The same study found that *Ae. triseriatus* fed blood from white-tailed deer had significantly shorter gonotrophic cycles, while mosquitoes fed blood from squirrels and chipmunks needed a longer time to complete their gonotrophic cycle. However, *Ae. triseriatus* fed blood from squirrels and chipmunks experienced significantly higher fecundity than *Ae. triseriatus* fed blood from white-tailed deer (Mather and DeFoliart, 1983).

Mahmood et al (2004) fed *Culex tarsalis* heparinized chicken blood (heparin sodium added to the blood to prevent coagulation) with 6 $\log_{10}$ PFU Western equine encephalitis virus (WEEV)/ml for one group and 4 $\log_{10}$ PFU WEEV/ml for the other group. The same study incubated mosquitoes at 26°C. Findings indicate fecundity was significantly lower in the WEEV infected group (133 and 134 eggs/female in the 6 $\log_{10}$ PFU/ml and 4 $\log_{10}$ PFU/ml respectively versus 213 eggs/female in the uninfected group); however, fertility was significantly higher in the WEEV infected groups (Mahmood et al., 2004). A study performed by Richards et al. (2010) looked at the effects of blood meal source (live chicken, chicken blood in Alsever's [AC]

solution, defibrinated bovine blood, and citrated bovine blood) on the reproduction of *Culex pipiens quinquefasciatus* (Diptera: Culicidae) at 28°C. Results show *Cx. p. quinquefasciatus* fed AC exhibited significantly higher fecundity during the first blood feeding when compared with all other groups (Richards et al., 2010). The fastest hatch times were found in the groups fed live chicken blood or AC (Richards et al., 2010). In the same study, *Cx. p. quinquefasciatus* fed defibrinated bovine blood and citrated bovine blood experience the lowest fecundity and fertility in the first blood feeding (Richards et al., 2010). Scientists should consider that different blood sources could affect colony propagation and estimates of vector competence. This knowledge is important to improve laboratory studies evaluating risk of mosquito populations for virus transmission. We expect the findings of this study to improve methods for mosquito colony propagation and inform research using artificial blood delivery methods to assess vector competence.

Chapter 5 – Conclusion

Little research has been performed on the Pan American expansion of DEN over the past decade and the increase of international travel has aided its status as the most prevalent vector borne disease in humans. As range expansion of vectors and international travel continues to increase, DEN epidemics will continue to occur. The US is at risk of DENV establishment due to international travel, climate change, and presence of competent vectors (Moore and Mitchell, 1997; MMRW, 2010; WHO, 2012). The current lack of serological testing, standardized diagnoses, and efficient reporting systems impede DEN risk assessment (Gubler, 2002; Guzman, 2003; Gubler 2012). These increased risks are important to public health due to the economic burden of lost work time and associated medical costs (MMWR, 2010; Rothman, 2013, Gubler 1998). The geographic ranges of the four DENV serotypes are expected to expand with international travel as humans are the primary reservoirs (Gubler, 2012). This increases the likelihood of multi-serotype epidemics that could impact public health. Risk assessments showing the impact of travel on importation of DENV are essential to understand the role of human travel in pathogen spread.

Laboratory vector competence studies should consider methodological differences, such as blood source, that may impact estimates of vectorial capacity. No previous study has examined the effects of blood meal source and EIT on life table characteristics and vector competence for DENV in *Ae. albopictus*. While studies on the effects of EIT *Culex* spp. infected with WNV and SLEV show increases in vector competence at higher EITs (Richards et al., 2007; Richards et al., 2012), a study on *Ae. albopictus* has shown that EIT had no effect on DENV vector competence when comparing effects on virus titers (Alto and Bettinardi 2013).

Alto and Bettinardi (2013) show similar findings at 20°C, 25°C and 30°C compared to our results for infection and dissemination at 27°C and 30°C.

Artificial blood delivery systems are often used to propagate mosquito colonies as this method is cost effective compared to maintaining live animals (Turell, 1988; Richards et al., 2012). Commercially available blood also replaces the time required to maintain live animals which are expensive to keep and subject to government regulation (Turell, 1988). However, laboratories that employ artificial blood delivery systems must also consider the effect of the blood source on the life table characteristics (and possibly vector competence) of the mosquitoes (Richards et al., 2012). Future studies with additional EITs, mosquito populations and species, and blood meal sources will contribute to the overall knowledge of mosquito colony propagation and studies determining transmission risk.

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Appendix

Table 1. Serologically Positive Cases of Dengue in 52 Pan American Countries from 2001-2012

		2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
North America	Canada	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	United States	96	29	40	10	98	143	488	199	178	648	283	545
Central America	Belize	3	41	NA	2	380	9	40	23	1457	2178	469	1948
	Costa Rica	9237	12251	19669	9408	37798	12124	26440	7160	6946	31773	13854	22243
	El Salvador	1093	18307	7436	13344	15290	22088	12476	5774	15040	22406	20836	41793
	Guatemala	4516	7599	6750	6352	6341	2428	5886	3230	10438	17045	2565	9547
	Honduras	9077	32269	16559	19971	18843	8436	33508	18941	15291	66814	8297	15554
	Mexico	6210	9844	5018	8202	16862	27287	48436	31154	249763	57971	67918	164947
	Nicaragua	2104	2157	2799	1035	1735	1350	1415	1424	17140	6261	11888	30499
	Panama	1545	711	293	373	4000	4300	3402	2287	6811	1243	3882	1329
South America	Argentina	11	214	135	3284	34	181	173	40	26612	1185	213	2043
	Bolivia	176	892	6548	7390	4443	2040	7332	3181	84047	5191	26681	42704
	Brazil	416067	780644	341902	112928	203789	346550	559954	734384	528883	1004392	764032	565510
	Chile (Only Easter Island)		636	NA	NA	NA	3	28	25	27	NA	1	34
	Colombia	55437	76996	52588	27523	30475	36471	43227	26732	51543	157152	33207	49361
	French Guiana	2830	280	2178	3147	4365	15930	661	460	11330	4350	667	1372

	Guyana	60	202	33	47	178	118	201	324	994	1468	1093	2189
	Ecuador	10919	5833	10319	6165	12131	6044	10587	1894	4489	1042	7659	18995
	Paraguay	38	1871	137	164	405	4271	28182	1953	6143	13553	42945	33063
	Peru	23329	8875	3637	9774	6358	5531	6907	10278	8813	18392	29810	29994
	Suriname	760	1104	285	375	2853	285	41	24	120	113	409	781
	Uruguay	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Venezuela	83180	37676	26996	30693	42198	39860	80646	48048	65869	123967	31551	49044
Caribbean	Cuba	11432	3011	NA	NA	75	NA	28	NA	70	NA	NA	NA
	Dominican Republic	3592	3194	6163	2476	2860	6143	9628	4333	8292	11519	2339	9665
	Puerto Rico	5233	2906	3735	3288	5701	3043	11012	3384	6651	21298	5654	12877
	American Virgin Islands	NA	NA	NA	NA	NA	NA	73	NA	NA	NA	NA	NA
	Anguilla	25	5	2	NA	NA	NA	NA	9	NA	1	9	9
	Antigua and Barbuda	20	5	NA	NA	NA	NA	NA	17	NA	3	7	10
	Aruba	NA	25	NA	173	NA	5	NA	NA	845	1415	3027	667
	Bahamas	NA	NA	180	1	NA	NA	NA	1	NA	8	7000	5
	Barbados	1043	740	557	349	320	1	NA	1	55	2917	745	1445
	Bermuda	NA	NA	NA	NA	2	2	NA	NA	NA	2	1	NA
	British Virgin Islands	23	NA	NA	NA	1	NA	6	15	65	9	939	214
	Cayman Islands	NA	1	1	NA	1	NA	9	1	NA	8	2	53
	Curacao	NA	NA	NA	NA	265	NA	NA	NA	NA	1723	1555	721

	Dominica	5	NA	NA	4	11	19	111	80	2	635	40	29
	Grenada	12	84	17	7	NA	22	NA	6	23	125	87	75
	Guadaloupe	NA	93	495		3364	2948	3266	2234	2234	41100	824	1032
	Haiti	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	240
	Jamaica	39	90	52	9	46	79	1448	359	70	2827	408	4670
	Martinique	4471	392	791	NA	6083	4086	5082	586	1378	37100	275	1269
	Montserrat	1	1	1	NA	NA	NA	NA	2	NA	NA	3	1
	Netherlands Antilles	NA	NA	NA	NA	NA	NA	NA	NA	NA	852	939	121
	St. Bartolome	NA	NA	NA	NA	NA	NA	NA	NA	805	NA	23	32
	St. Kitts & Nevis	89	20	2	4	NA	1	NA	49	2	19	47	1
	St. Lucia	292	51	5	11	1	NA	39	98	18	74	585	33
	St. Martin	NA	NA	NA	NA	NA	NA	NA	NA	1698	2450	168	253
	St. Vincent and the Grenadines	3	125	3	4	8	5	2	6	10	133	47	193
	Trinidad & Tobago	2244	6246	2289	546	411	37	47	2366	24	2497	1243	2473
	Turks & Caicos Islands	NA	NA	2	1	1	NA	NA	NA	NA	NA	24	16

Table 2: Serologically Positive Cases of Dengue in 18 Pan American Countries from 2001-2012*

World Rank	Pan American Rank	Country Name	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012
1	1	Canada	0	0	0	0	0	0	0	0	0	0	0	0
2	2	Mexico		1,2,3		1,2,3,4	1,2,3	1	1,2,3,4	1,2,3	1,2,3,4	1,2,3	1,2,3,4	1,2,3,4
7	3	Brazil	1,2,3	1,2,3	1,2,3	1,2,3	1,2,3	1,2,3	1,2,3	1,2,3	1,2,3	1,2,3,4	1,2,3,4	1,2,3,4
15	4	Venezuela	1,2,3,4	2,3,4	1,2,3	1,2,3,4	1,2,3,4	1,2,3,4	1,2,3,4	1,2,3,4	1,2,3,4	1,2,3,4	1,2,3,4	1,2,3,4
16	5	Columbia	1,2,4	1,3,4	1,2,3	1,2,3,4	1,2,3,4	1,2,3,4	1,2,3,4	1,2,3,4	1,2,3	1,2,3,4		
18	6	Argentina		1,3	1,2,3	3	2	2,3	2,3	1	1	1,2,3		2,3
25	7	Dominican Republic		2	2	2,4		1,2	1,2,3,4		1,2,4	1,2,4	2	2
26	8	Bahamas			2,4							1,2	1	
28	9	Guatemala	2,4	2,3,4	1,2,3,4	1,2,3,4	1,2,3,4	1,2,4	1,2,4	1,2	2,4	1,2,3,4	1,2	1,2,3
29	10	Jamaica							2,4	3		2		
31	11	Ecuador	1,2	1,2,3	3	3	2	3	3		1,3	1,2,3	1,2	2,4
33	12	Peru	1,2,3,4	1,3	1,2,3	1,2,3	1,2,3,4	3	1,2,3,4	1,3,4	1,2,3,4	1,2,3,4	1,2,3,4	1,2,3,4
34	13	Costa Rica	2	1,2	1,2	1,2	1	1,2	1	1,2	1,2	1,2,3	1,2,3	1,2,3
36	14	Trinidad and Tobago	2,3	2,3	3		2,3	2,3	3	2,3	2	2	1,4	
38	15	Chile		1					1	1	1,4		1	
39	16	El Salvador	2	1,2,3,4	2,4	1,2,4	2,4	1,2,4	1,2		1,2,3,4	1,2	1,2,3,4	1,2,3
41	17	Honduras	2,3,4	2,4	1,2,4	1,2,4	1,2,4		1,2,4	2,4	1,2	1,2,3,4	1,2	1,2
44	18	Panama	2	2	2	1,2,3	1,2		3	3	1,3	1,3	1,2,3	1,2,3

*Note: Of the top 50 countries in the world that travel to the US, 18 are from Pan America. This table shows those 18 countries.

Table 3: Description of Variables

Variable	*Calculations*
Fecundity	$= Number\ of\ eggs\ laid\ per\ female$
Fertility	$= \frac{Number\ of\ eggs\ hatched\ per\ female}{Number\ of\ eggs\ laid\ per\ female}$
Oviposition Time	$=$*Mean number of days to oviposit*
Hatch Time	$= Mean\ number\ of\ days\ to\ hatch$
Infection Rate	$= \frac{\#\ with\ DENV-positive\ bodies}{\#\ of\ blood\ fed\ mosquitoes}$
Dissemination Rate	$= \frac{\#\ with\ DENV-positive\ legs}{\#\ with\ DENV\ positive\ bodies}$
Horizontal Transmission Rate	$= \frac{\#\ with\ DENV\ -positive\ saliva}{\#\ with\ DENV\ -positive\ bodies}$
Transovarial Transmission Rate	$= \frac{\#\ of\ DENV\ -\ positive\ pools\ of\ offspring}{\#\ of\ pools\ of\ offspring}$

Table 4: Effects of Blood Source and EIT on Fecundity, Fertility, Oviposition Time and Hatch Time

Results of analysis of variance (PROC ANOVA) of effects of blood source and EIT on fecundity, fertility, oviposition time and hatch time. Significant values in bold type.

	DENV-infected group			**Uninfected group**		
Effect	***F***	**df num., denom.**	***P***	***F***	**df num., denom.**	***P***
			Fecundity			
Blood source	0.769	1,7	0.383	7.596	1,7	**0.007**
EIT	0.410	1,7	0.524	0.038	1,7	0.845
Blood source*EIT	0.539	1,7	0.465	0.454	1,7	0.501
			Fertility			
Blood source	0.030	1,7	0.863	7.089	1,7	**0.009**
EIT	3.536	1,7	0.063	0.086	1,7	0.769
Blood source*EIT	0.070	1,7	0.792	1.541	1,7	0.216
			Oviposition time			
Blood source	13.471	1,7	**0.00**	0.106	1,7	0.745

EIT	0.230	1,7	0.633	0.636	1,7	0.427
Blood source*EIT	0.776	1,7	0.381	1.994	1,7	0.161
			Hatch time			
Blood source	0.067	1,7	0.797	0.701	1,7	0.404
EIT	0.058	1,7	0.811	4.937	1,7	**0.029**
Blood source*EIT	0.297	1,7	0.587	0.132	1,7	0.717

Table 5: Effects of Status of Infection and Blood Source on Fecundity, Fertility, Oviposition Time and Hatch Time

Results of analysis of variance (PROC ANOVA) of effects of status of infection (infected or not infected) and blood source on fecundity, fertility, oviposition time and hatch time. Significant values in bold type.

Effect	*F*	df num., denom.	*P*
		Fecundity	
Blood source	1.002	1,7	0.318
Infection status	15.328	1,7	**0.000**
Blood source*Infection status	5.594	1,7	**0.019**
		Fertility	
Blood source	2.830	1,7	0.094
Infection status	0.568	1,7	0.452
Blood source*Infection status	3.574	1,7	0.060
		Oviposition time	
Blood source	5.058	1,7	**0.026**
Infection status	0.212	1,7	0.646

Blood source*Infection status	3.077	1,7	0.081
	Hatch time		
Blood source	0.647	1,7	0.422
Infection status	11.008	1,7	**0.001**
Blood source*Infection status	0.304	1,7	0.582

Table 6: Effects of Blood Source and EIT on Dissemination Rate, Horizontal Transmission Rate, Vertical Transmission Rate, Body Titer, Leg Titer and Saliva Titer at 14 and 21 Days Post Infection

Results of analysis of variance (PROC ANOVA) of effects of blood source and EIT on dissemination rate, horizontal transmission rate, vertical transmission rate, body titer, leg titer, and saliva titer at 14 and 21 days post infection. Significant values in bold type.

	14 Days Post Infection			**21 Days Post Infection**		
Effect	***F***	**df num., denom.**	***P***	***F***	**df num., denom.**	***P***
			Infection rate			
Blood source	n/a	n/a	n/a	n/a	n/a	n/a
EIT	n/a	n/a	n/a	n/a	n/a	n/a
Blood source*EIT	n/a	n/a	n/a	n/a	n/a	n/a
			Dissemination rate			
Blood source	2.188	1,3	0.145	0.882	1,3	0.362
EIT	0.088	1,3	0.768	0.882	1,3	0.362
Blood source*EIT	0.788	1,3	0.379	0.882	1,3	0.362
			Horizontal transmission rate			
Blood source	3.360	1,3	0.072	7.353	1,3	**0.016**

EIT	0.373	1,3	0.544	7.353	1,3	**0.016**
Blood source*EIT	0.373	1,3	0.544	0.294	1,3	0.596
			Vertical transmission rate			
Blood source	n/a	n/a	n/a	n/a	n/a	n/a
EIT	n/a	n/a	n/a	n/a	n/a	n/a
Blood source*EIT	n/a	n/a	n/a	n/a	n/a	n/a
			Body titer			
Blood source	9.282	1,3	**0.004**	0.149	1,3	0.705
EIT	0.027	1,3	0.870	0.209	1,3	0.654
Blood source*EIT	0.098	1,3	0.756	3.427	1,3	0.084
			Leg titer			
Blood source	0.487	1,3	0.489	0.006	1,3	0.937
EIT	0.414	1,3	0.524	0.385	1,3	0.545
Blood source*EIT	1.217	1,3	0.276	1.073	1,3	0.318
			Saliva titer			
Blood source	5.570	1,3	**0.000**	0.246	1,3	0.641
EIT	0.054	1,3	0.628	0.019	1,3	0.895

Blood source*EIT	0.239	1,3	0.316	n/a	0	n/a

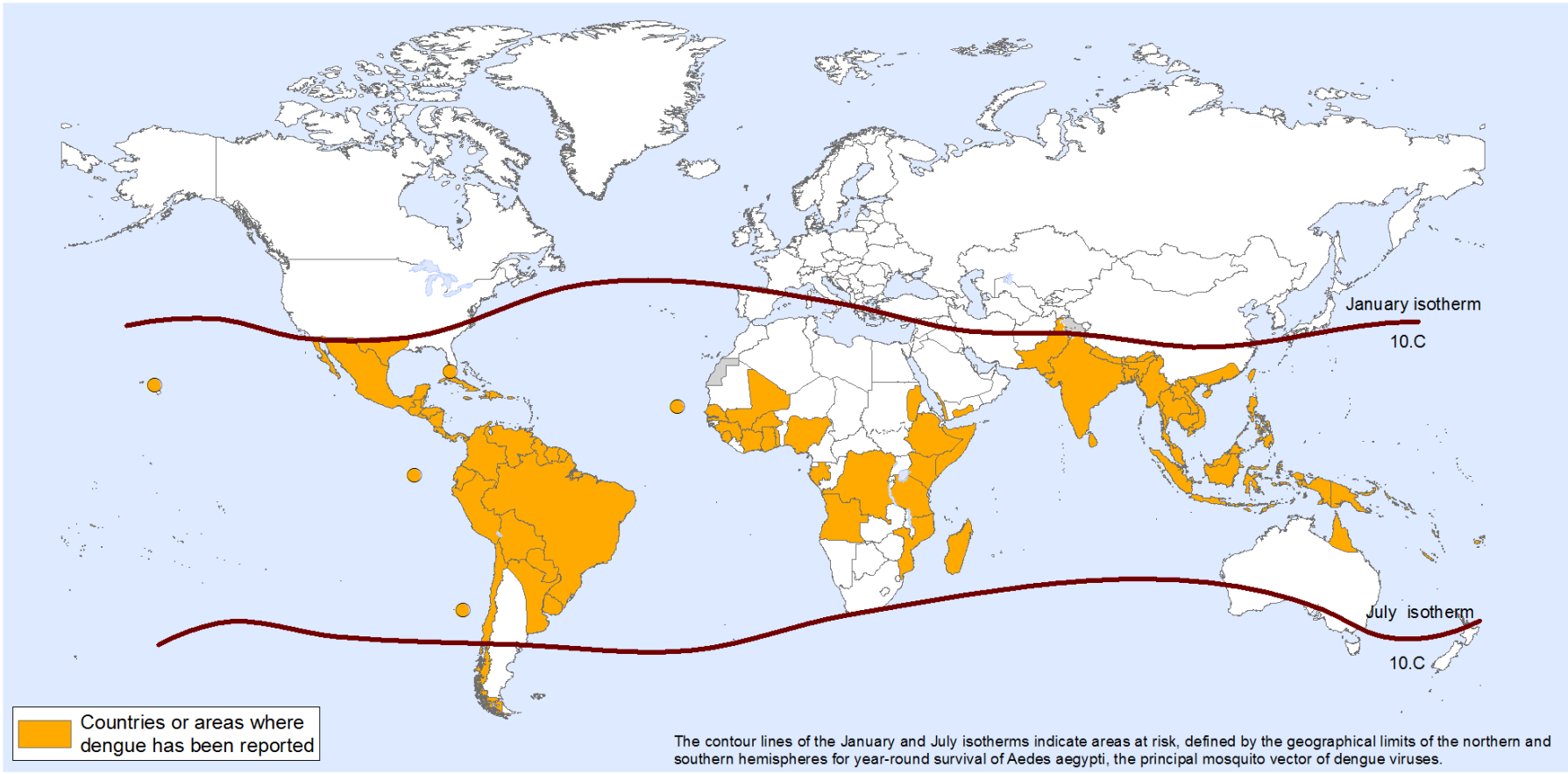
Note: Infection rate was 100% across all groups at 14 and 21 days post infection. Therefore statistical analysis were performed on infection rate.

Table 7: Chi- Square Analysis of Survival of Uninfected vs. DENV-Infected Mosquitoes

Significant values in bold type.

Temperature	**Test**	***F***	**df num., denom.**	***P***
27°C	Log Rank (Mantel-Cox)	7.86	3,4	**0.049**
	Breslow (Generalized Wilcoxon)	8.11	3,4	**0.044**
	Tarone-Ware	8.00	3,4	**0.046**
30°C	Log Rank (Mantel-Cox)	11.17	3,4	**0.011**
	Breslow (Generalized Wilcoxon)	11.06	3,4	**0.011**
	Tarone-Ware	11.14	3,4	**0.011**

Figure 1. Countries/Areas at Risk of Dengue Transmission



The boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted and dashed lines on maps represent approximate border lines for which there may not yet be full agreement.

Data Source: World Health Organization
Map Production: Health Statistics and Information Systems (HSI)
World Health Organization

World Health Organization

Figure 2. International Travel to the US from Pan American Regions

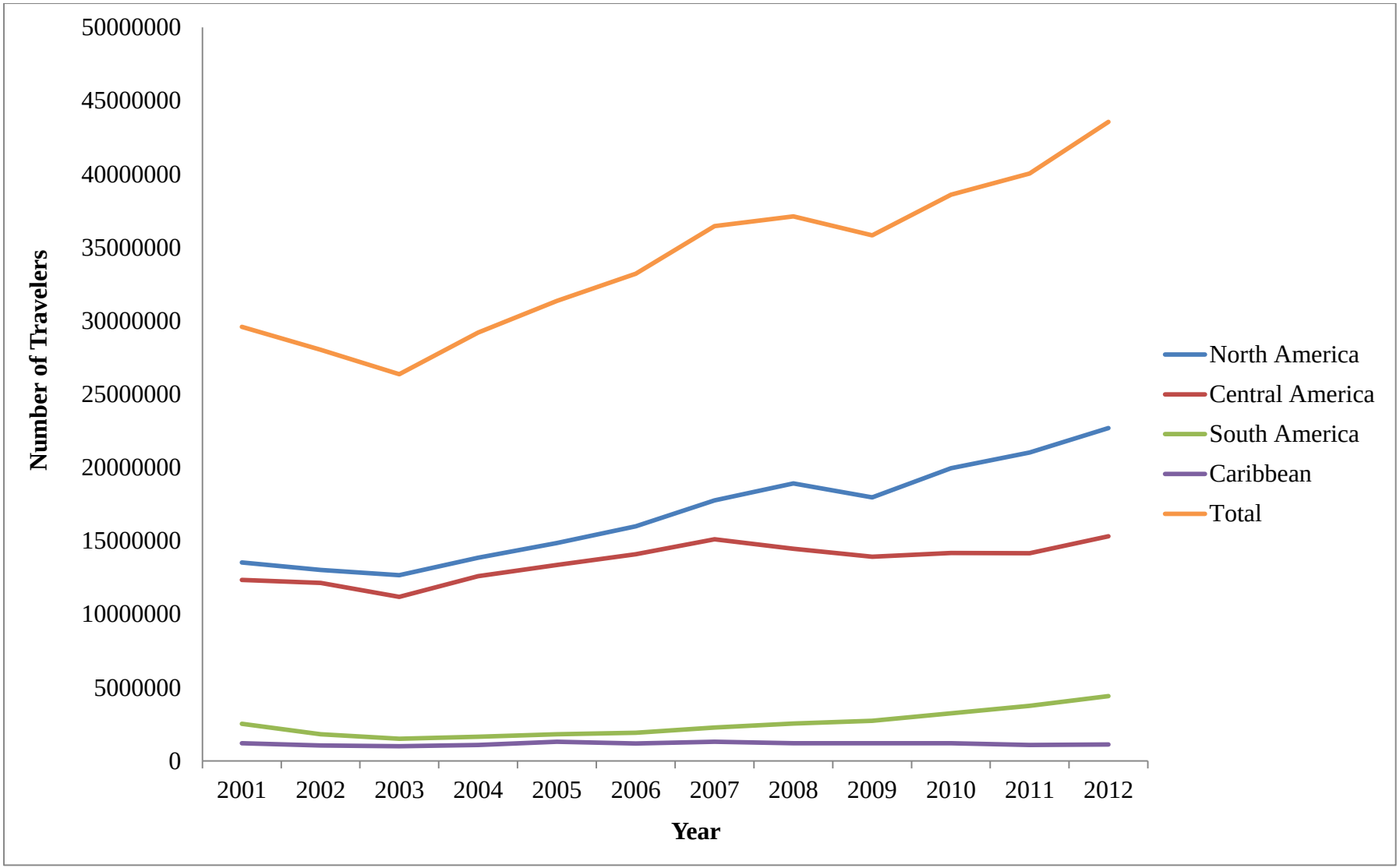


Figure 3. Serologically Positive Cases of Dengue Fever per Pan American Region

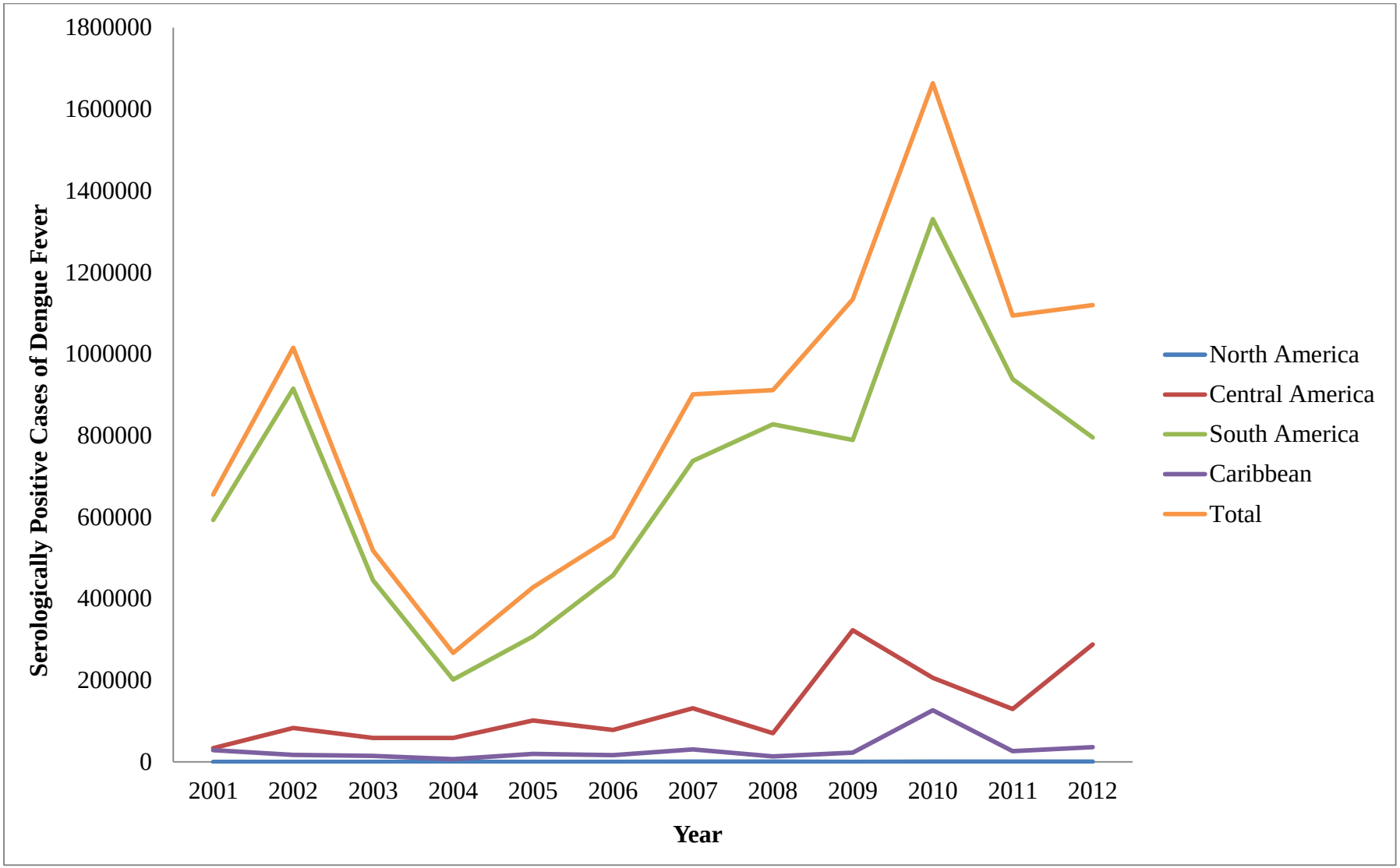


Figure 4. Incidence Rate of Dengue Infection in Pan American Regions

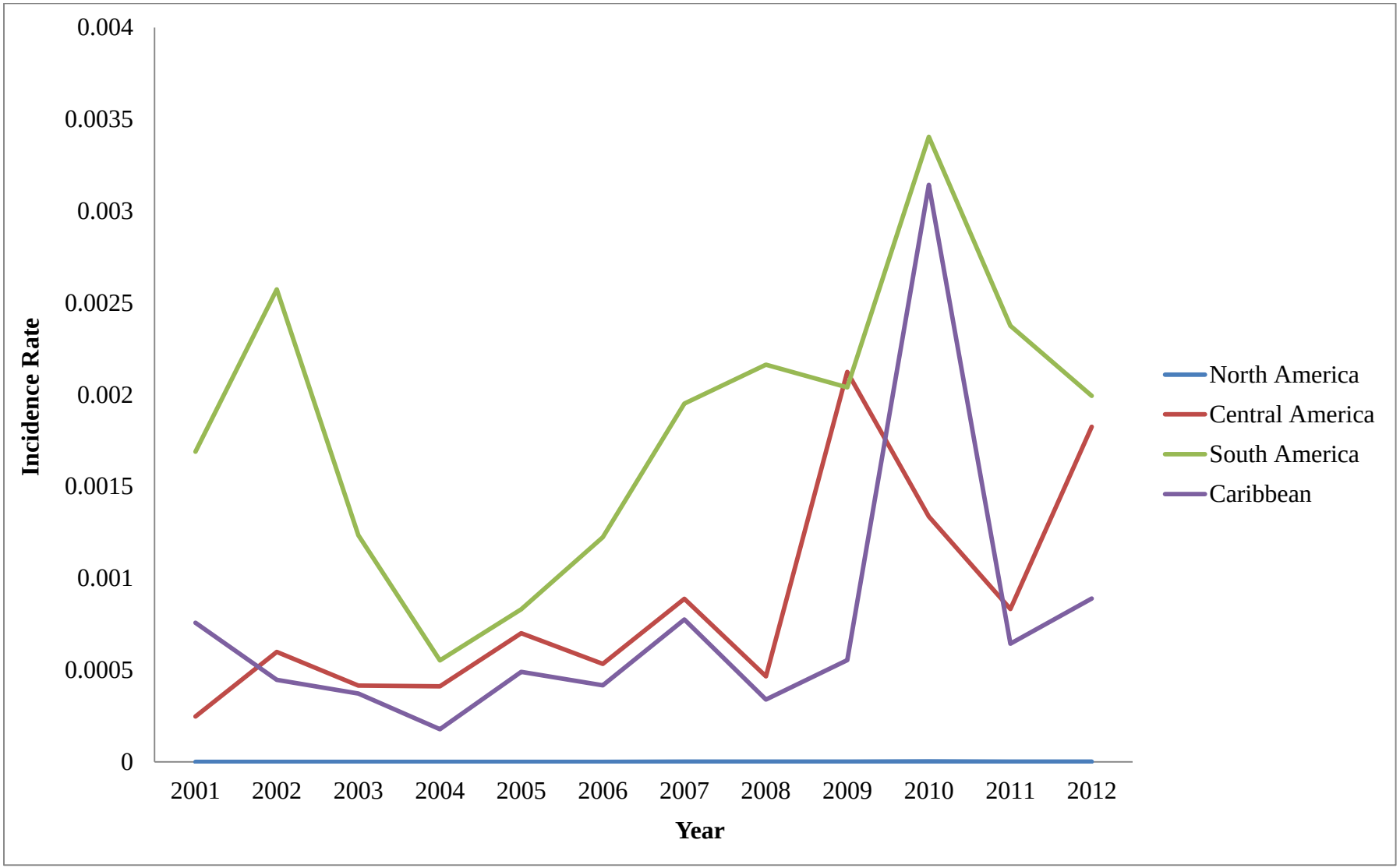


Figure 5. Potentially Infected Travellers Entering the US/Year by Pan American Region

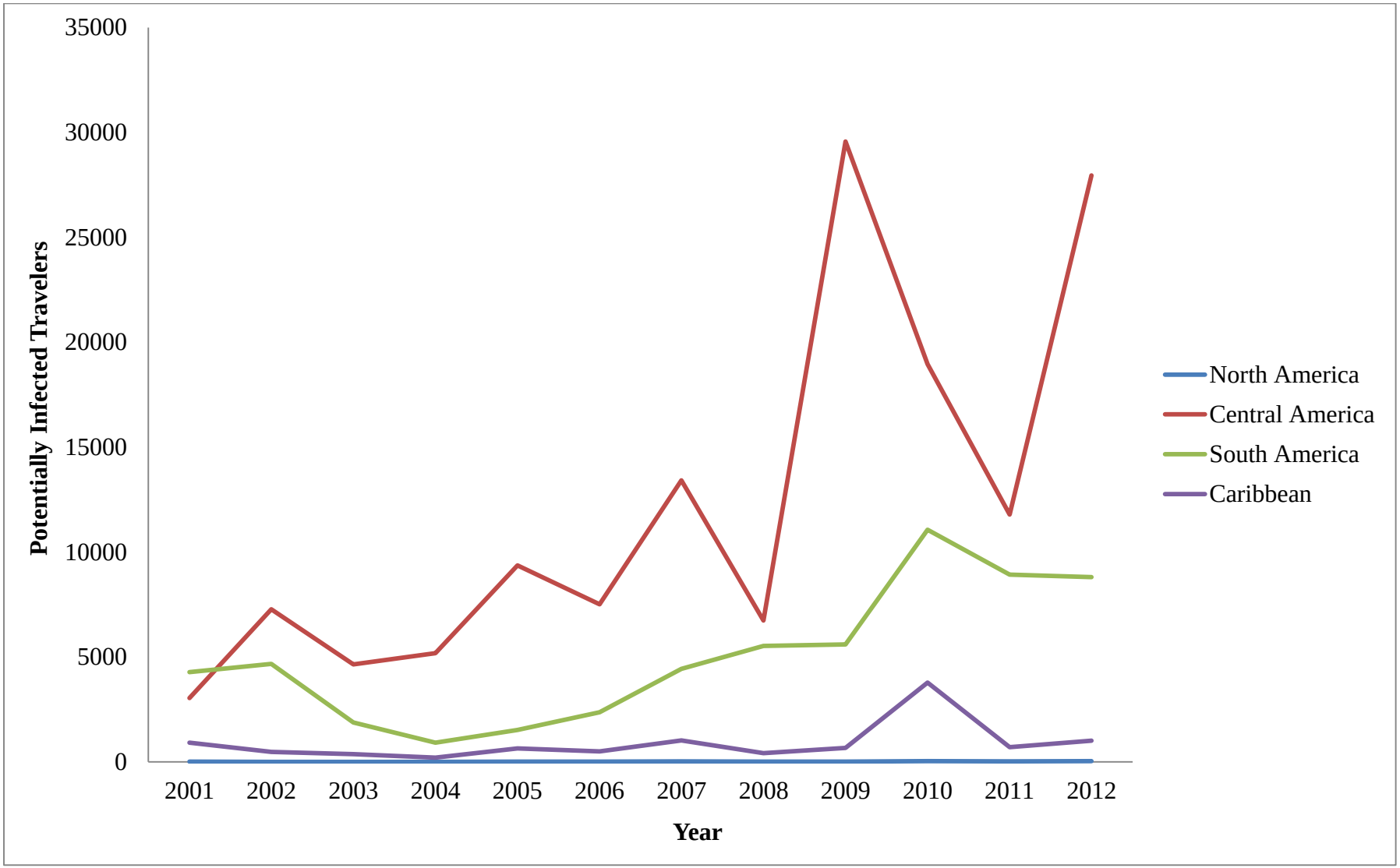
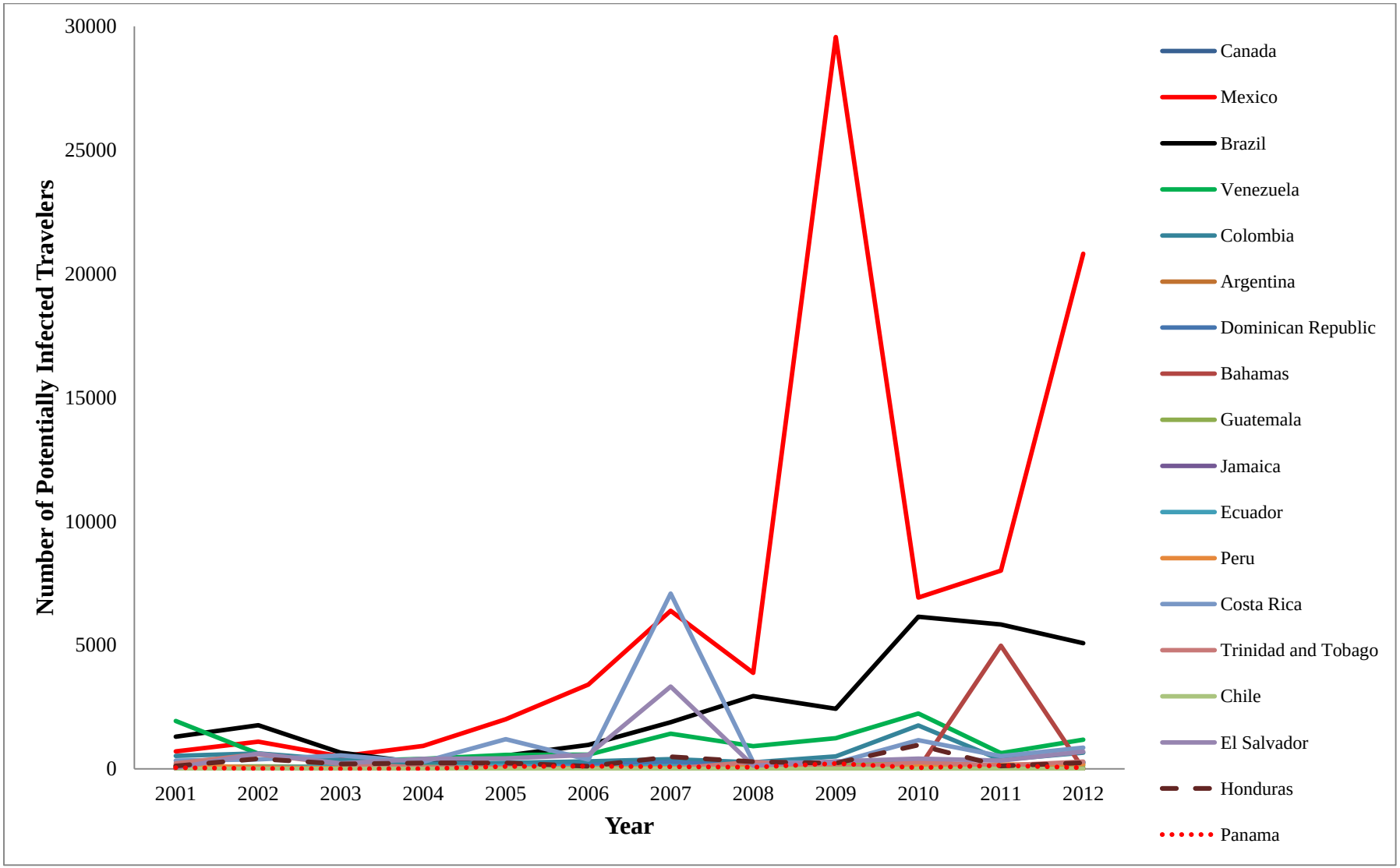


Figure 6. Number of Potentially Infected Pan American Travellers Entering the US/ Year



Note: Of the top 50 countries in the world that travel to the US, 18 are from Pan America. This graph shows those 18 countries.

Figure 7. Infection Rate throughout Pan America/ 100,000 People in 2004

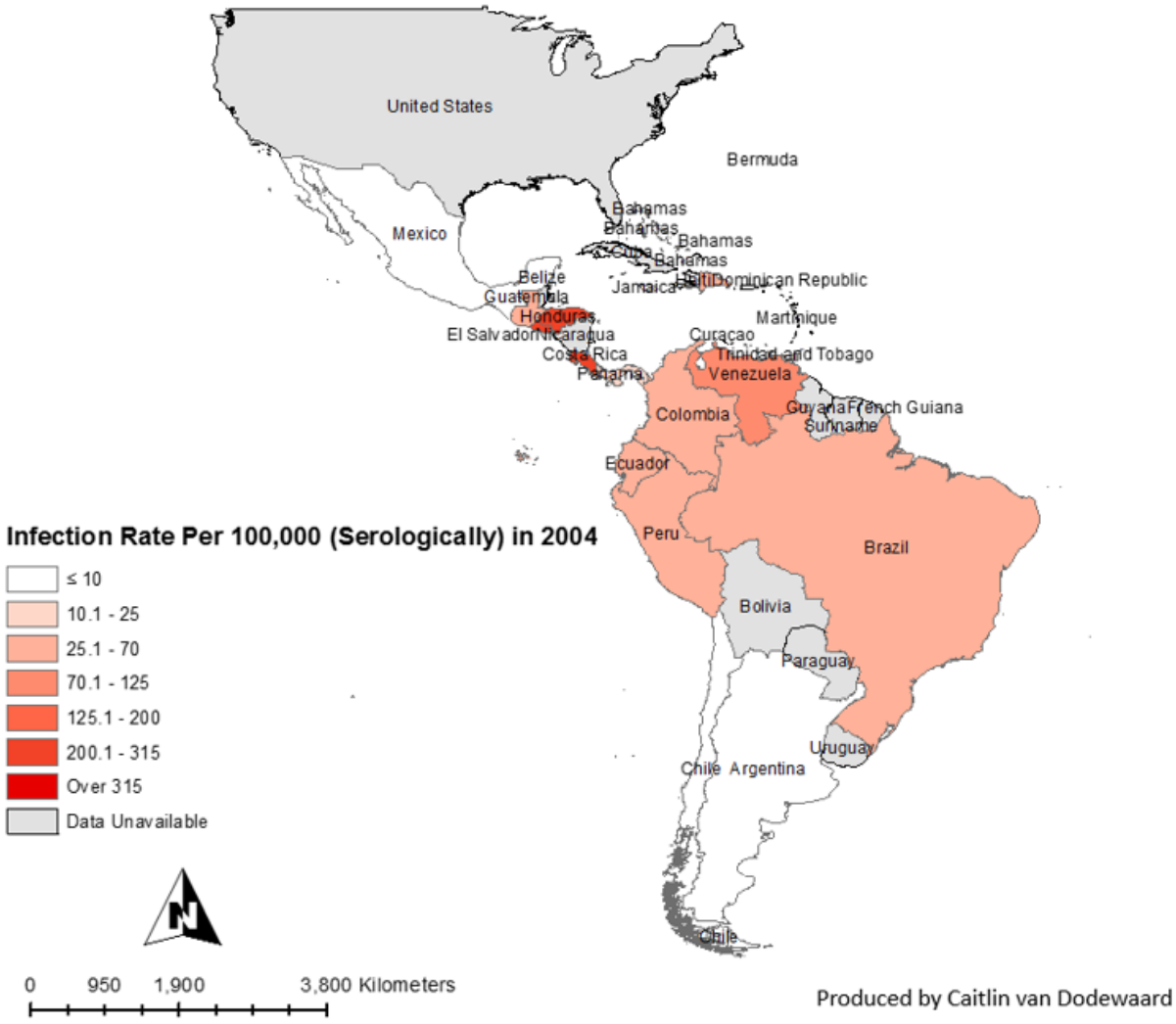


Figure 8. Infection Rate throughout Pan America/ 100,000 People in 2008

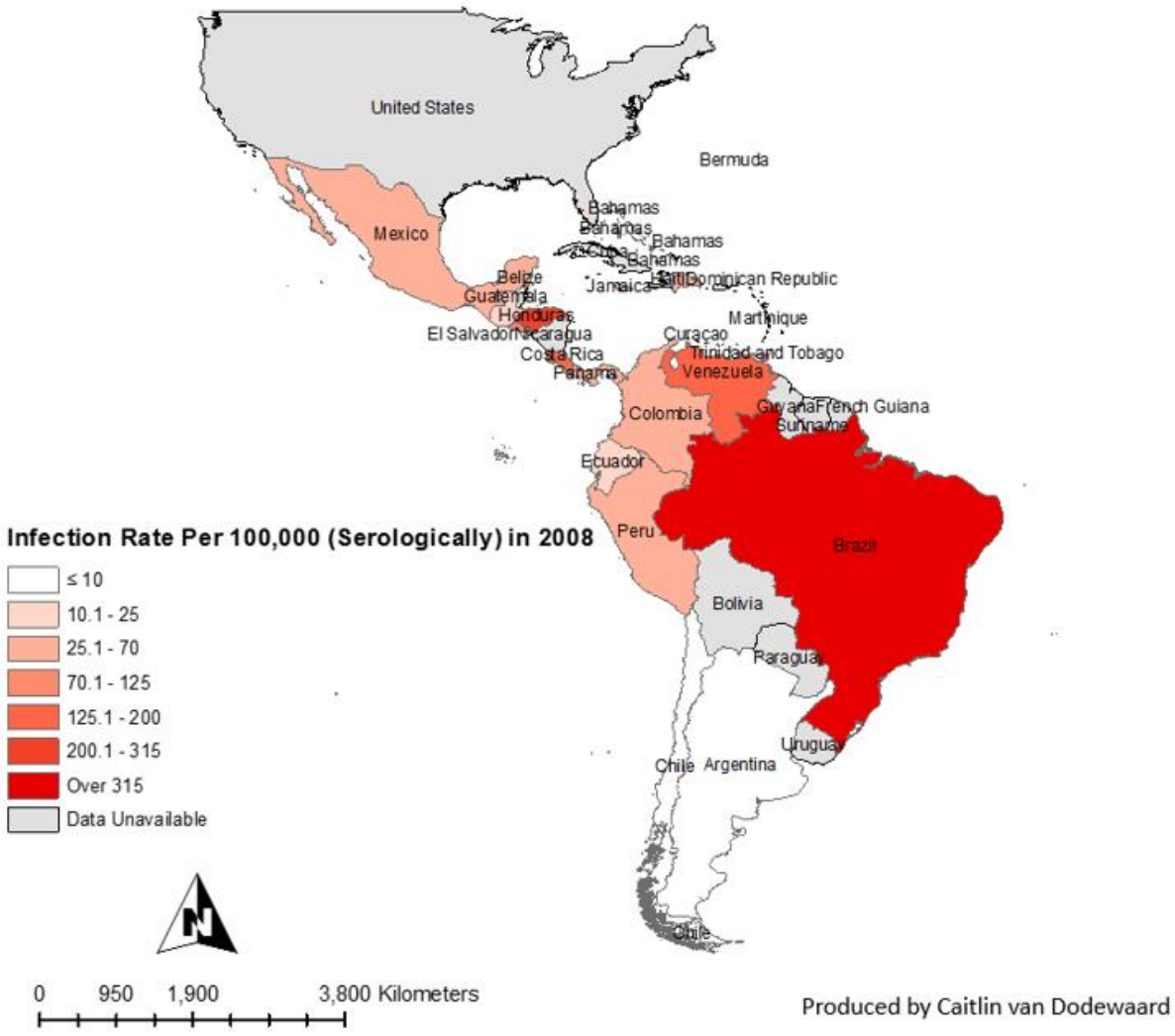


Figure 9. Infection Rate throughout Pan America/ 100,000 People in 2012

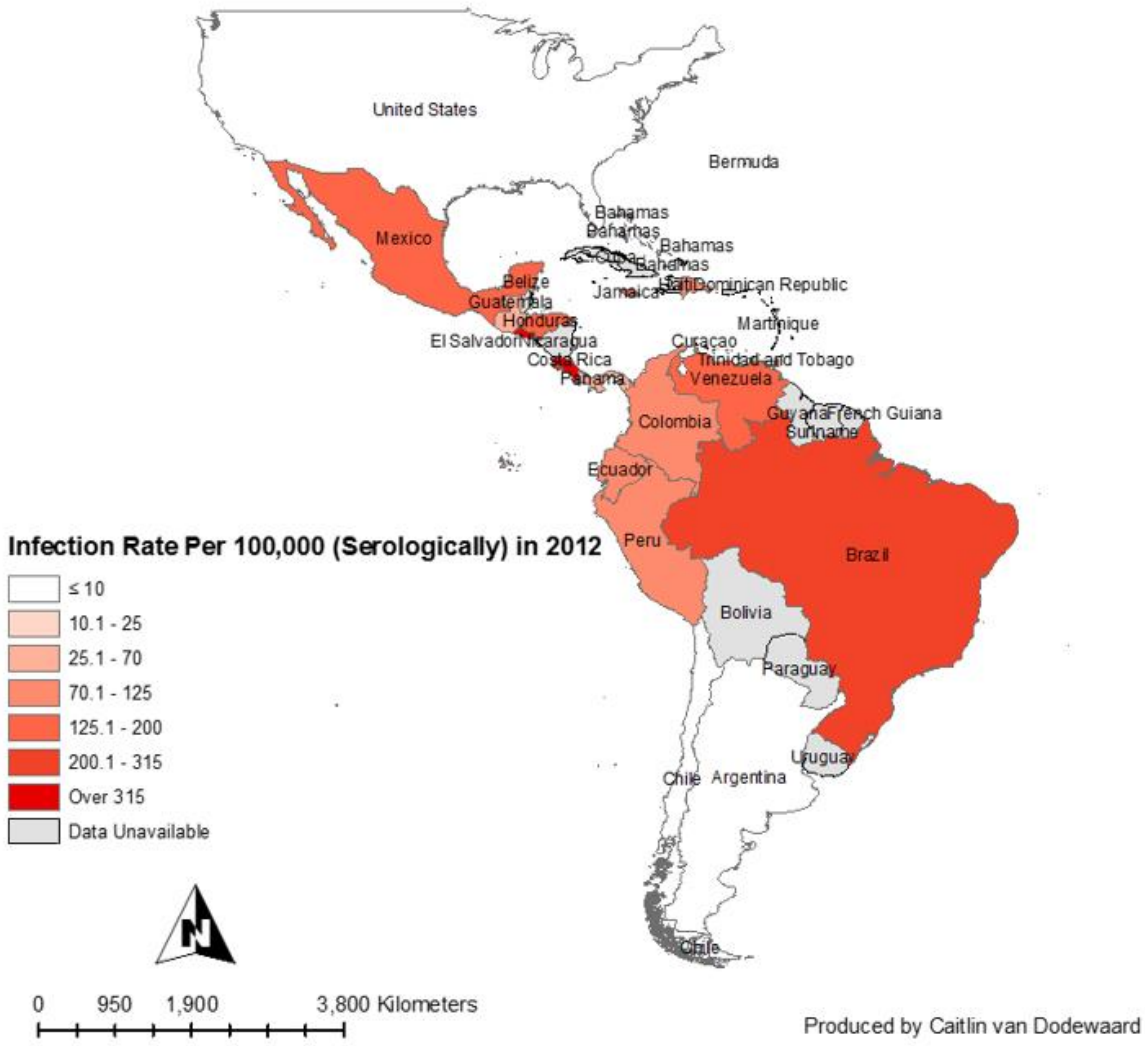


Figure 10. Study Design and Treatment Groups

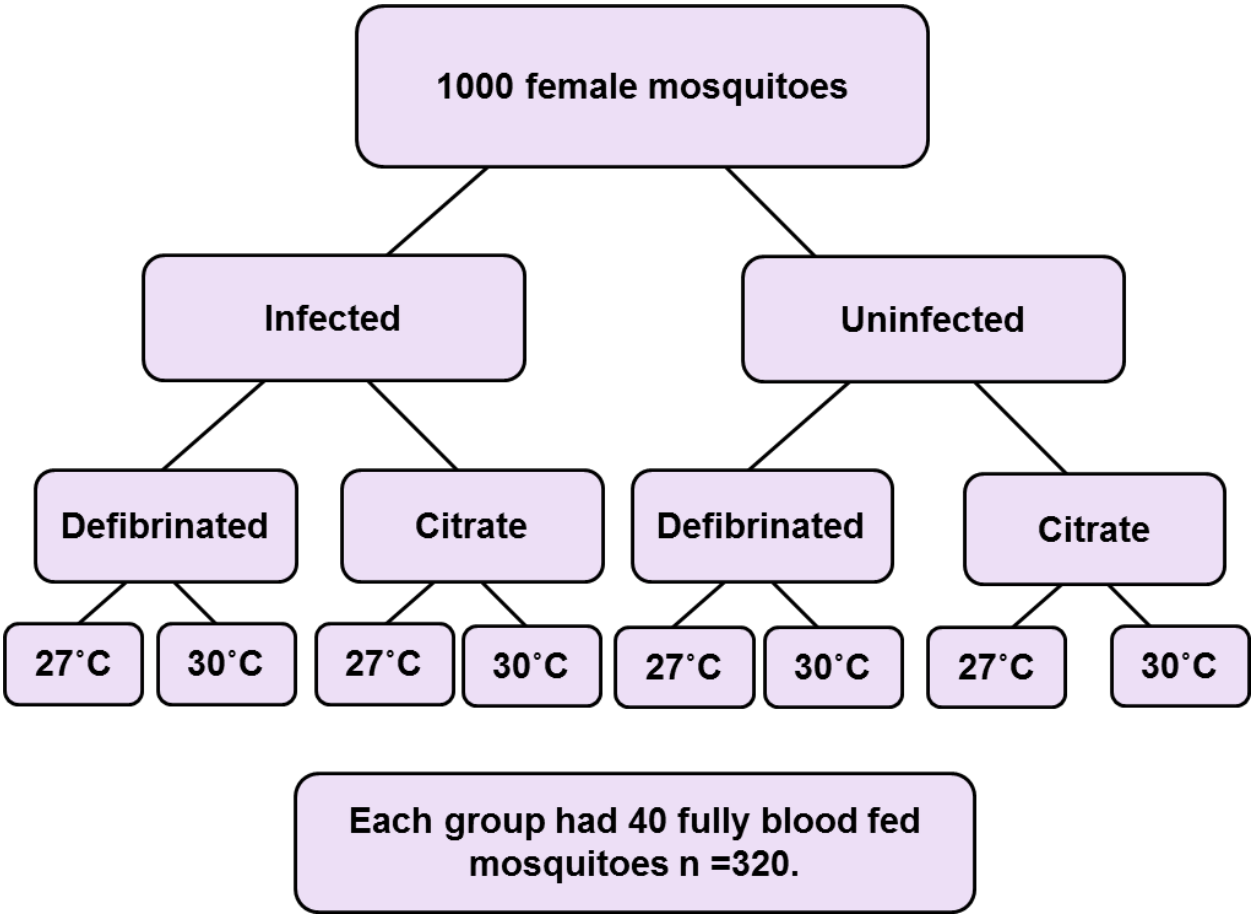
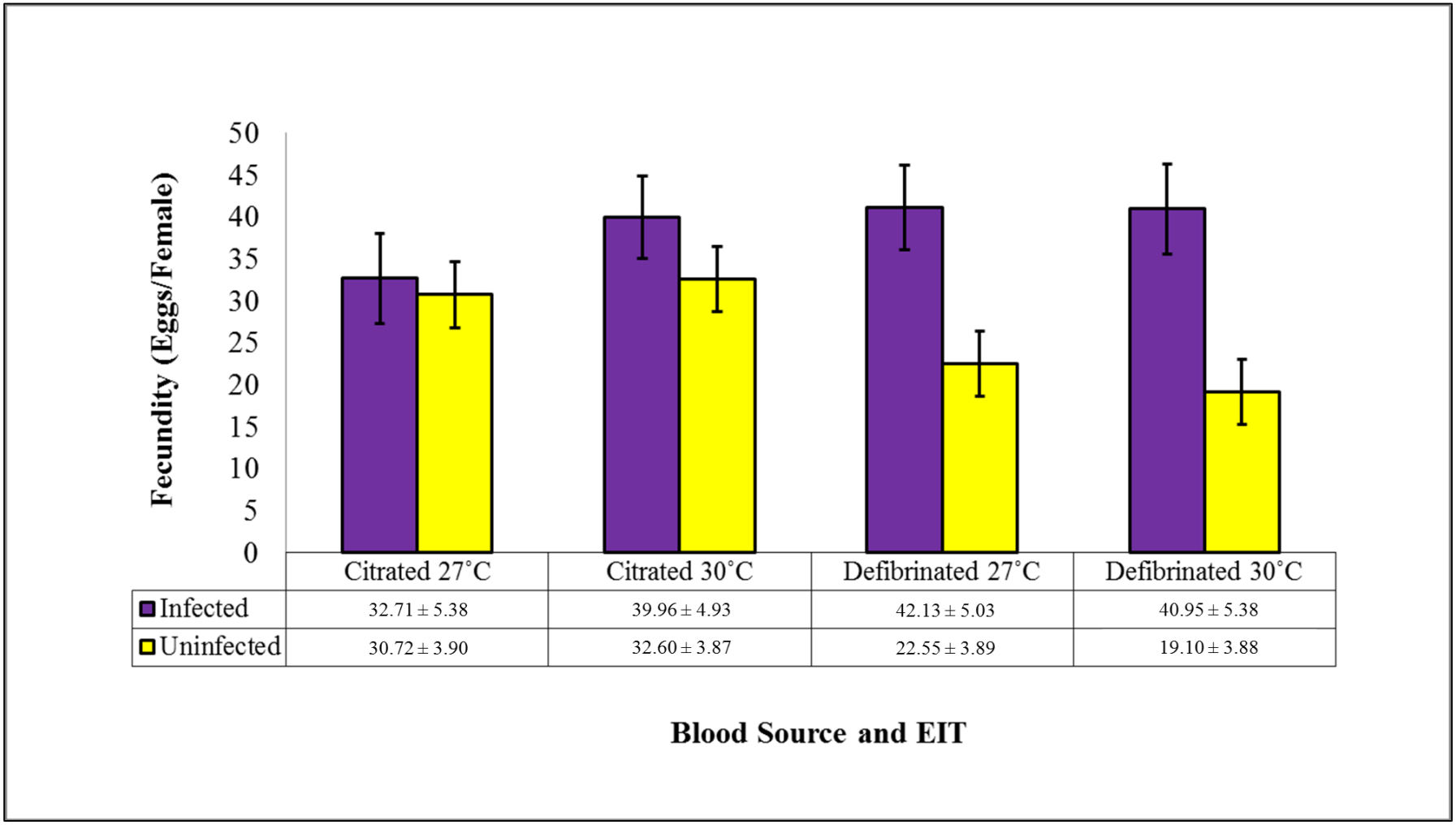
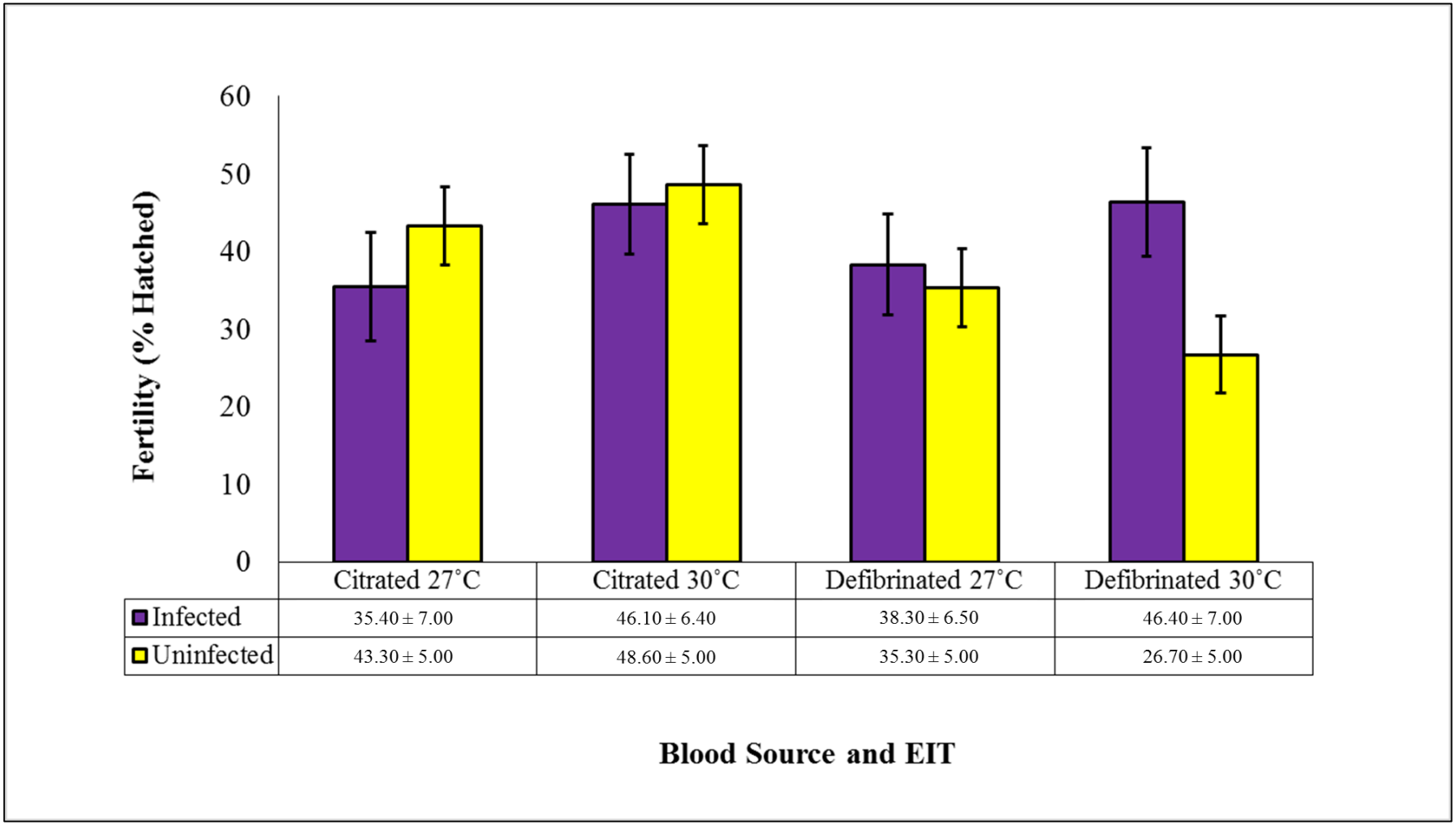


Figure 11. Effect of Blood Source and EIT on Fecundity



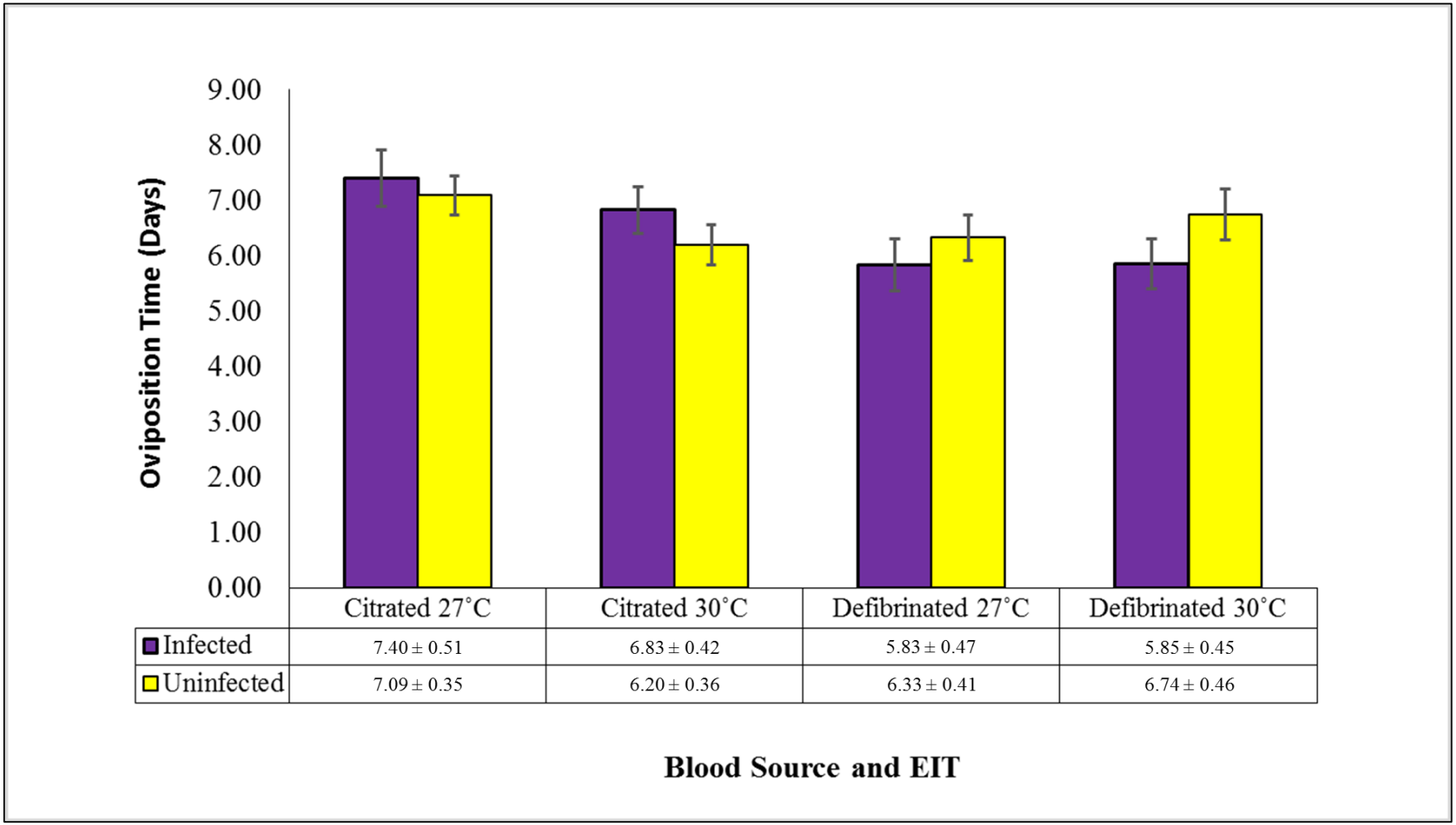
	Citrated 27˚C	Citrated 30˚C	Defibrinated 27˚C	Defibrinated 30˚C
Infected	32.71 ± 5.38	39.96 ± 4.93	42.13 ± 5.03	40.95 ± 5.38
Uninfected	30.72 ± 3.90	32.60 ± 3.87	22.55 ± 3.89	19.10 ± 3.88

Figure 12. Effect of Blood Source and EIT on Fertility



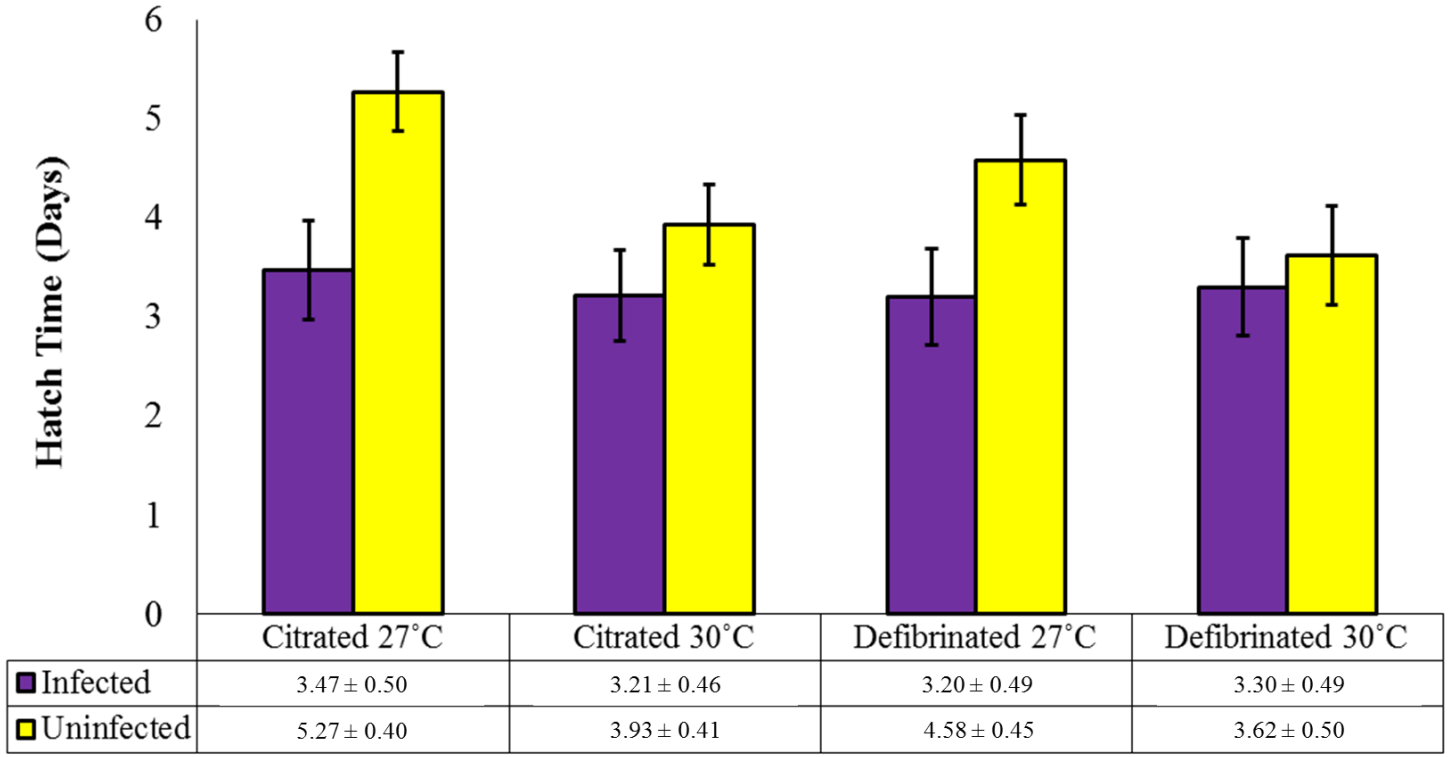
	Citrated 27˚C	Citrated 30˚C	Defibrinated 27˚C	Defibrinated 30˚C
Infected	35.40 ± 7.00	46.10 ± 6.40	38.30 ± 6.50	46.40 ± 7.00
Uninfected	43.30 ± 5.00	48.60 ± 5.00	35.30 ± 5.00	26.70 ± 5.00

Figure 13. Effect of Blood Source and EIT on Oviposition Time



	Citrated 27˚C	Citrated 30˚C	Defibrinated 27˚C	Defibrinated 30˚C
Infected	7.40 ± 0.51	6.83 ± 0.42	5.83 ± 0.47	5.85 ± 0.45
Uninfected	7.09 ± 0.35	6.20 ± 0.36	6.33 ± 0.41	6.74 ± 0.46

Figure 14. Effect of Blood Source and EIT on Hatch Time



	Citrated 27˚C	Citrated 30˚C	Defibrinated 27˚C	Defibrinated 30˚C
Infected	3.47 ± 0.50	3.21 ± 0.46	3.20 ± 0.49	3.30 ± 0.49
Uninfected	5.27 ± 0.40	3.93 ± 0.41	4.58 ± 0.45	3.62 ± 0.50

Figure 15. Effect of Blood Source and EIT on Infection, Dissemination and Transmission at 14 Days Post Blood Feeding

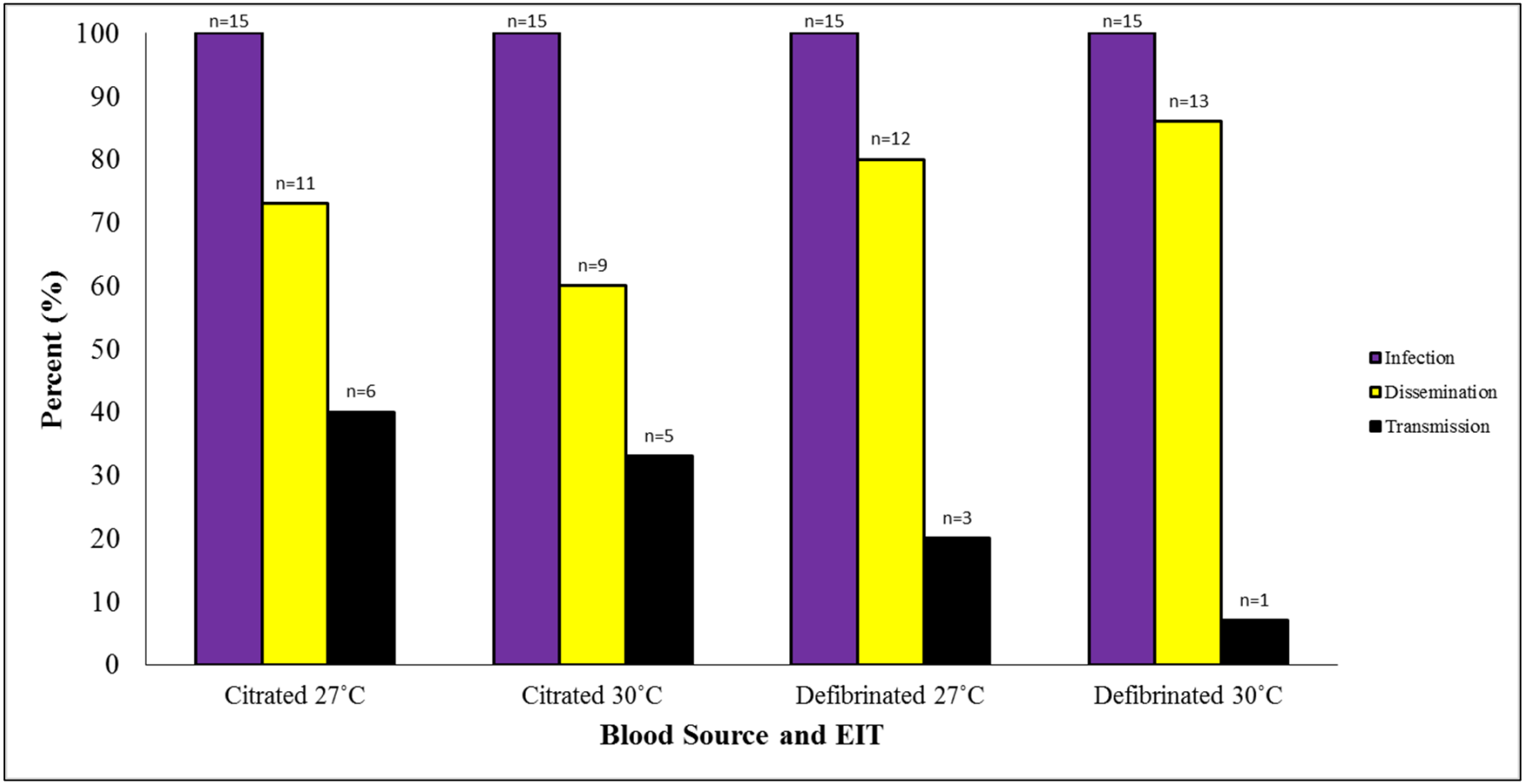


Figure 16. Effect of Blood Source and EIT on Infection, Dissemination and Transmission at 21 Days Post Blood Feeding

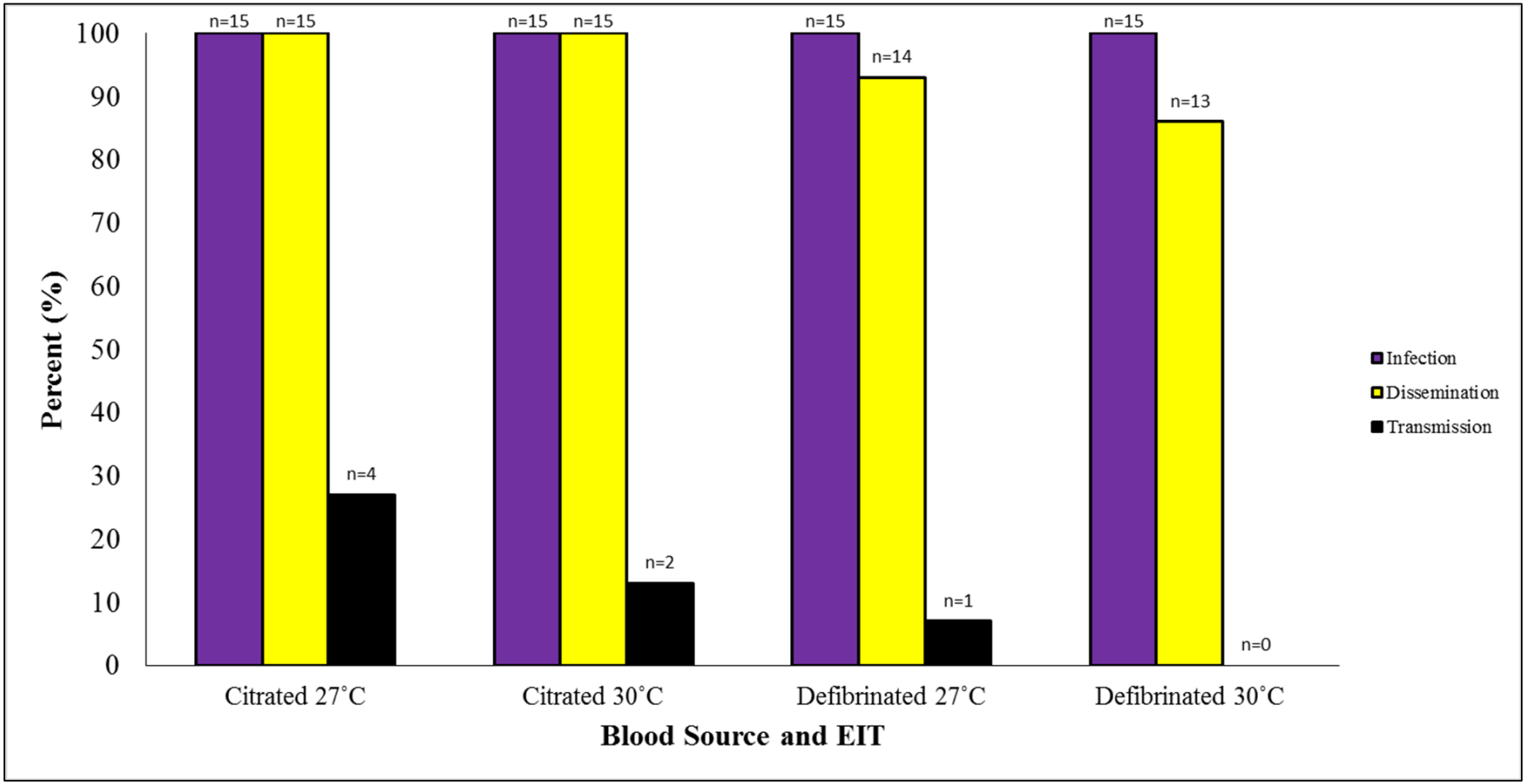
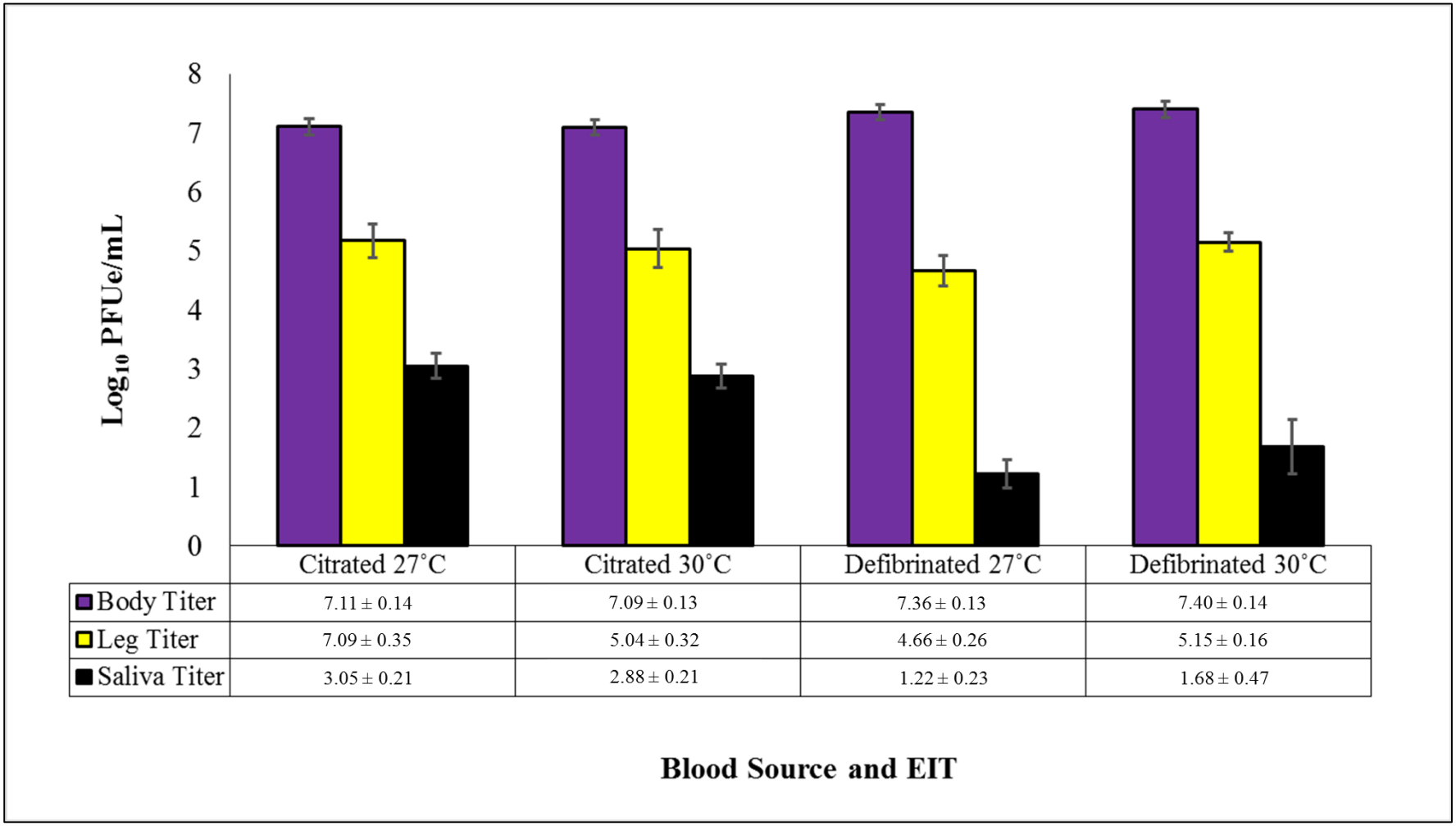
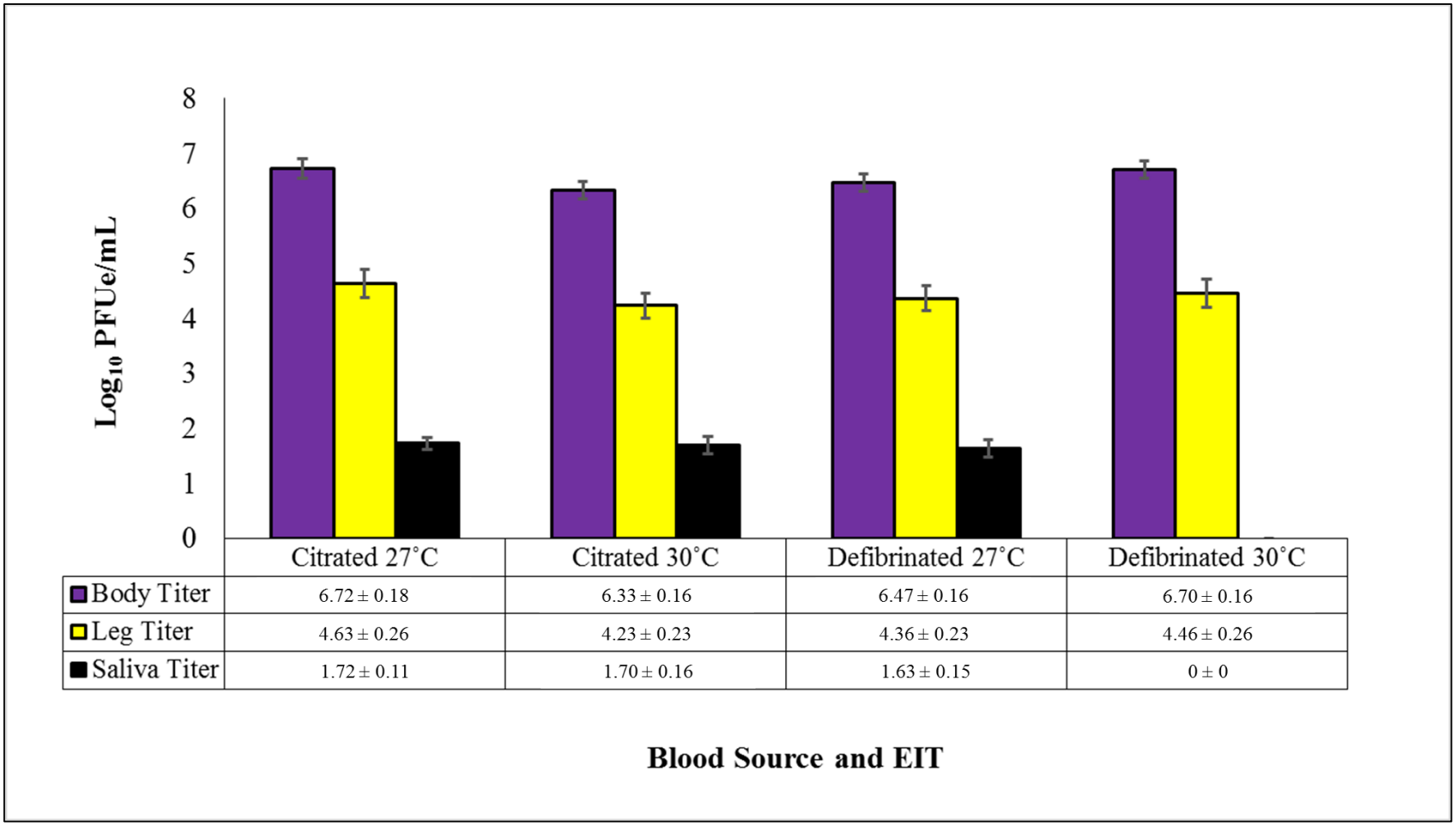


Figure 17. Effect of Blood Source and EIT on Body, Leg and Saliva Titers at 14 Days Post Feeding



	Citrated 27˚C	Citrated 30˚C	Defibrinated 27˚C	Defibrinated 30˚C
Body Titer	7.11 ± 0.14	7.09 ± 0.13	7.36 ± 0.13	7.40 ± 0.14
Leg Titer	7.09 ± 0.35	5.04 ± 0.32	4.66 ± 0.26	5.15 ± 0.16
Saliva Titer	3.05 ± 0.21	2.88 ± 0.21	1.22 ± 0.23	1.68 ± 0.47

Figure 18. Effect of Blood Source and EIT on Body, Leg and Saliva Titers at 21 Days Post Blood Feeding



	Citrated 27˚C	Citrated 30˚C	Defibrinated 27˚C	Defibrinated 30˚C
Body Titer	6.72 ± 0.18	6.33 ± 0.16	6.47 ± 0.16	6.70 ± 0.16
Leg Titer	4.63 ± 0.26	4.23 ± 0.23	4.36 ± 0.23	4.46 ± 0.26
Saliva Titer	1.72 ± 0.11	1.70 ± 0.16	1.63 ± 0.15	0 ± 0

Figure 19. Effect of Blood Meal Source and EIT (27°C) on Survival

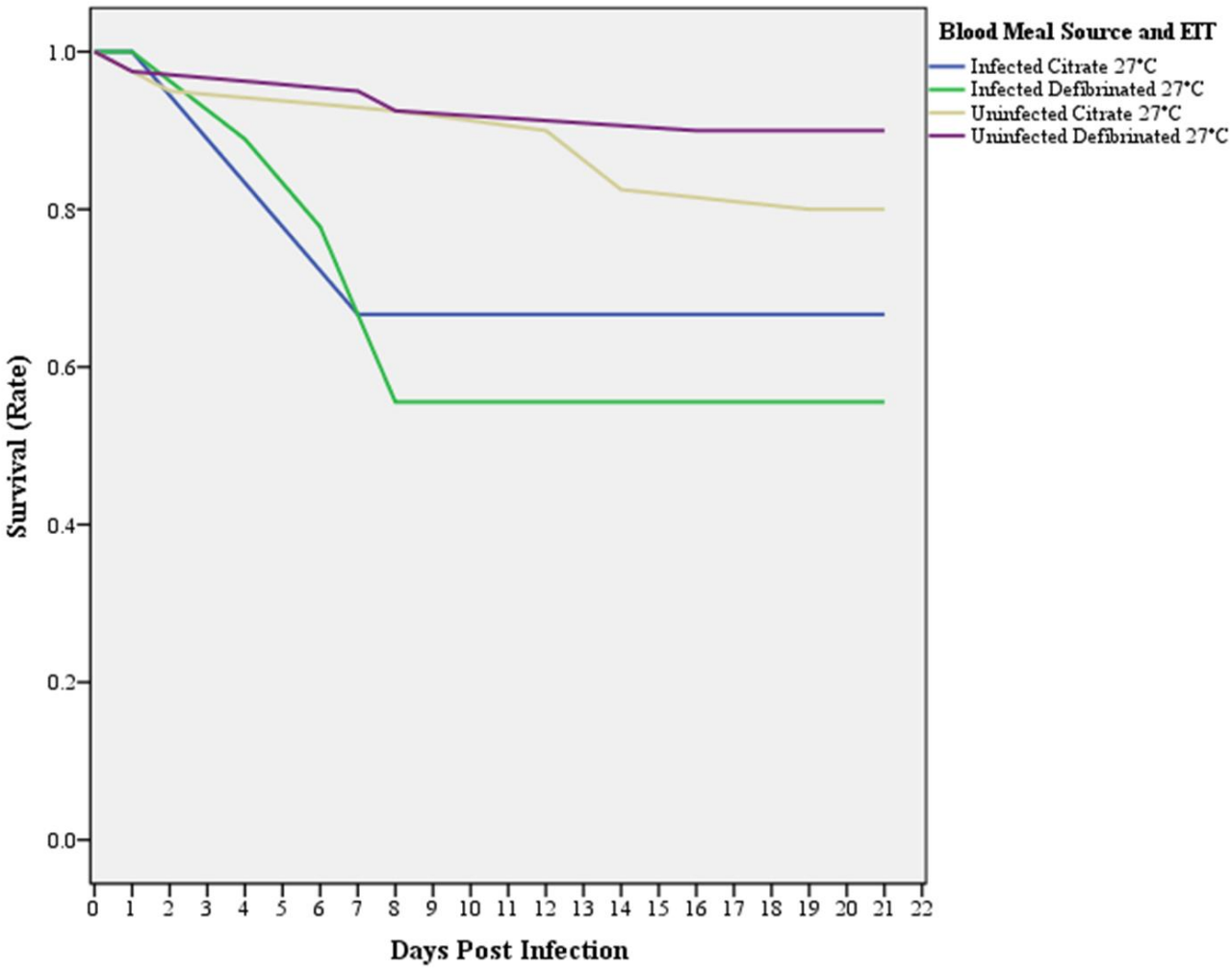


Figure 20. Effect of Blood Meal Source and EIT (30°C) on Survival