CHARACTERIZATION OF *AEDES (STEGOMYIA) AEGYPTI* AND *AEDES (STEGOMYIA) ALBOPICTUS* POPULATIONS AND CHIKUNGUNYA VIRUS PREVALENCE IN PUERTO BARRIOS, GUATEMALA

by

Lieutenant Jefferson M. Moody

Environmental Health Officer

United States Navy

Thesis submitted to the Faculty of the
Department of Preventive Medicine and Biostatistics
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In partial fulfillment of the requirements for the degree of
Masters of Science in Public Health 2016

[Dissertation approval form inserted here]

UNIFORMED SERVICES UNIVERSITY, SCHOOL OF MEDICINE GRADUATE PROGRAMS
Graduate Education Office (A 1045), 4301 Jones Bridge Road, Bethesda, MD 20814

May 11, 2016

MEMORANDUM FOR: **MARY T. BRUEGGEMEYER, MD, MPH**
JAMES C. DUNFORD, PhD
DAVID F. HOEL, PhD

SUBJECT: Appointment to the Final Master's Examination Committee for Jefferson M. Moody.

In accordance with the School of Medicine Graduate Program Guidelines, you are formally appointed to the Final Examination Committee of Jefferson M. Moody, Master of Science in Public Health student in the Department of Preventive Medicine & Biostatistics.

This Committee will evaluate the scope and qualities of the student's Dissertation and certify the document's acceptability.

In addition, the Committee will conduct an Oral Examination of the student's scientific knowledge, as well as his knowledge/ understanding of hir thesis research project and Dissertation. This Examination constitutes the Private Defense.

The Private Defense for Jefferson M. Moody will take place on Monday, May 16, 2016 at 13:00, in Room A2074.

Mary T. Brueggemeyer, MD, MPH is appointed Chair of the Examination Committee. She will oversee the Examination. Passage of the Private Defense will be by majority vote. If corrections to the Dissertation are needed, the Chair may oversee this process and sign-off after the corrections are made.

The Committee Chair should return the signed Final Examination/Private Defense Form and the Dissertation Approval Form to the Graduate Education Office as soon as each Form is completely signed.

GEO thanks you in advance for your participation in the SOM Graduate Program process.

[redacted]

Gregory P. Mueller, Ph.D.
Associate Dean for Graduate Education

UNIFORMED SERVICES UNIVERSITY, SCHOOL OF MEDICINE GRADUATE PROGRAMS
Graduate Education Office (A 1045), 4301 Jones Bridge Road, Bethesda, MD 20814

FINAL EXAMINATION/ PRIVATE DEFENSE FOR THE DEGREE OF MASTER OF SCIENCE IN PUBLIC HEALTH IN THE DEPARTMENT OF PREVENTIVE MEDICINE AND BIOSTATISTICS

Name of Student: Jefferson M. Moody

Date of Examination: May 16, 2016

Time: 13:00

Place: Room A2074

DECISION OF EXAMINATION COMMITTEE MEMBERS:

	PASS	FAIL
[redacted]	✓	___

Mary T. Brueggemeyer, MD, MPH
DEPARTMENT OF PREVENTIVE MEDICINE & BIOSTATISTICS
Committee Chairperson

[redacted]	✓	___

James C. Dunford, PhD
DEPARTMENT OF NAVY AND MARINE CORPS PUBLIC HEALTH CENTER
Thesis Advisor

[redacted]	✓	___

David F. Hoel, PhD
DEPARTMENT OF PREVENTIVE MEDICINE & BIOSTATISTICS
Committee Member

UNIFORMED SERVICES UNIVERSITY, SCHOOL OF MEDICINE GRADUATE PROGRAMS
Graduate Education Office (A 1045), 4301 Jones Bridge Road, Bethesda, MD 20814

DISSERTATION APPROVAL FOR THE MASTER IN SCIENCE IN PUBLIC HEALTH DISSERTATION IN THE DEPARTMENT OF PREVENTIVE MEDICINE AND BIOSTATISTICS

Title of Thesis: "Characterization of Aedes (Stegomyia) aegypti and Aedes (Stegomyia) albopictus Populations and Chikungunya Virus Prevalence in Puerto Barrios, Guatemala"

Name of Candidate: Jefferson M. Moody
Master of Science in Public Health Degree
May 16, 2016

THESIS AND ABSTRACT APPROVED:

DATE:

[redacted] 21 June 2016

Mary T. Brueggemeyer, MD, MPH
DEPARTMENT OF PREVENTIVE MEDICINE & BIOSTATISTICS
Committee Chairperson

[redacted] 21 June 2016

James C. Dunford, PhD
DEPARTMENT OF NAVY AND MARINE CORPS PUBLIC HEALTH CENTER
Thesis Advisor

[redacted] 21 June 2016

David F. Hoel, PhD
DEPARTMENT OF PREVENTIVE MEDICINE & BIOSTATISTICS
Committee Member

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DEDICATION

To Jennifer, Taylor and Grayson.

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Moody, Jefferson Milvar

June 21, 2016,

ABSTRACT

Characterization of *Aedes* (*Stegomyia*) *aegypti* and *Aedes* (*Stegomyia*) *albopictus* populations and chikungunya virus prevalence in Puerto Barrios, Guatemala.

Lieutenant Jefferson M. Moody, Masters of Science in Public Health, 2016

Thesis directed by: Col Mary T. Brueggemeyer, MC, SFS, USAF; CAPT David F. Hoel, PhD, Entomologist, MSC, USN; LCDR James C. Dunford, PhD, Entomologist, MSC, USN

Chikungunya virus has rapidly spread throughout tropical regions of the Western Hemisphere since making the transoceanic leap into the Caribbean in late 2013. *Aedes aegypti* and *Aedes albopictus* are competent vectors of chikungunya (RNA alphavirus) and the geographical range of both species has been expanding. Formal documentation of *Aedes albopictus* as an established species has been difficult to obtain for several Central American countries due to the lack of adequate surveillance and ability to conduct field research in austere environments; thus, the prevalence and distribution of *Aedes albopictus* in many Central American countries is largely unknown and it has not been formally implicated in transmitting chikungunya virus in Guatemala. Adult mosquitoes were collected from 25-30 September 2015 in the port city of Puerto Barrios utilizing BG-Sentinel™ traps to determine the presence of chikungunya virus infection in *Aedes aegypti* and *Aedes albopictus* populations. Multiple sampling points were randomly selected in categorized urban or rural sites and collected mosquitoes were placed in sterile collection tubes and preserved using

RNALater®. Sample pools were homogenized and RNA was extracted for reverse transcription polymerase chain reaction (RT-PCR) analysis.

A total of 106 mosquitoes (*Anopheles, Culex,* and *Aedes spp.)* were collected in urban and rural areas during the sample period, 37% of those mosquitoes were *Aedes* spp. Nine mosquitoes were identified as *Aedes albopictus*. Fifteen sample pools of mosquitoes consisting of one to eight mosquitoes per pool tested negative for chikungunya virus. Distribution of mosquito species as well as proportions of infected mosquitoes in urban and rural environments could not be determined due to the sample size being less than the 88 sample pools suggested for power. During the sample period Puerto Barrios was experiencing a chikungunya outbreak and public health personnel were conducting aggressive adulticiding campaigns to combat the health threat in the community. While the sample collection was under powered and resulted in no detection of virus, mosquito sampling methods in the field and pathogen testing protocols at Navy Environmental and Preventive Medicine Unit 2 (NEPMU-2) laboratories yielded procedures for follow-on disease surveillance studies. In addition, collection of *Aedes albopictus* from several rural and urban sites in Puerto Barrios 20 years after an initial finding reported by Ogata and Samayoa in1996 showed that it is likely established in Puerto Barrios. With the emergence of Zika virus in 2015, an RNA virus also transmitted by *Aedes* mosquitoes, protocols developed during this study may enable additional DoD laboratories such as NEPMU-2 to test for infected mosquitoes in collected sample pools and this field project has trained Puerto Barrios Ministry of Health (MOH) personnel to collect and process *Aedes* mosquitoes for virus sampling.

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CHAPTER 1: Introduction

BACKGROUND

Chikungunya virus is an enzootic virus found in tropical and subtropical regions around the world including Africa, the Indian Ocean Islands, and South and Southeast Asia. The virus has recently spread to the Americas and is now considered a panzootic disease. Chikungunya was first isolated from a febrile patient during an outbreak on the Makonde Plateau in the southern province of Tanzania (formerly Tanganyika) in 1952–53. The disease gets its name from the Kimakonde vernacular language of Tanzania and Mozambique; the word chikungunya means ‘‘that which contorts or bends up’’ and translates in Swahili to ‘‘the illness of the bended walker’’[1].

Chikungunya is a mosquito-borne viral infection of humans that previously was confined to regions in Central Africa; however, during this century, the virus has shown surprising potential for geographic expansion as it invaded other countries including those in more temperate regions. Humans serve as the primary reservoir during epidemic periods while several vertebrates have been implicated as potential reservoirs during inter-epidemic periods, including non-human primates, rodents, birds, and some small mammals [2]. There are currently no vaccines available and no specific treatment for chikungunya infections; the main control strategy for chikungunya remains preventive control of mosquito populations and personal protective measures [3].

The disease shares most clinical signs with dengue, and can be misdiagnosed in areas where dengue is common. The majority of infected people will develop some symptoms; chikungunya is unusual among arboviruses in that typically >70% of seropositive humans develop disease [4]. This is in stark contrast to the majority of arboviral agents capable of

causing illness in humans such as Eastern equine encephalitis, St. Louis encephalitis, West Nile virus and dengue virus, among others, in which the vast majority of infected people remain asymptomatic [5]. Chikungunya disease seldom results in death, but the symptoms can be severe, disabling and last for months or years [5]. The disease is characterized by fever, headache, myalgia, rash, and joint pain, especially of the small joints (wrists, fingers). Although most symptoms resolve, some patients have joint pain that can continue for years and can be so severe that they adopt a bent or stooping posture. The Swahili term “illness of the bended walker” accurately describes chikungunya disease as highly debilitating, and large epidemics can have severe economic consequences; thus, there is an urgent need for continued research into the pathogenesis, prevention and treatment of these infections [6].

The prevalence of *Ae. albopictus* in Guatemala and many of the Central American countries is largely unknown, and this species of mosquito has not been formally implicated in transmitting chikungunya virus in Guatemala. In the United States, *Ae. albopictus* may have displaced *Ae. aegypti* [7] throughout much of its original Southeastern U.S. range. *Aedes albopictus* was not recognized in Guatemala [8] and on Hispaniola (in the Dominican Republic) until the early 1990s [2] and it has only recently been reported in Haiti in 2012 [3]. This likely reflects lack of entomological surveillance in most Central/South American and Caribbean countries. It is unclear how both species will coexist in similar ecological niches in these locations and what epidemiological implications this may have on the prevalence of chikungunya transmission. *Aedes albopictus* has been demonstrated a competent vector in numerous other chikungunya virus outbreaks in the Indian Ocean region and Europe. The adaptation of chikungunya virus to *Ae. albopictus* mosquitoes likely contributed to the dramatic spread of chikungunya virus in the Eastern Hemisphere [6]. Evidence of chikungunya virus transmission

through *Ae. albopictus* in Guatemala may also elucidate the potential emergence for locally transmitted chikungunya virus in the U.S. Mid-Atlantic region in which *Ae. albopictus* is ubiquitous among areas of significant human population density.

Since 2004, chikungunya virus has caused millions of cases of disease in the Indian Ocean basin and has emerged in new areas including Europe, the Middle East, and the Pacific region [6]. The mosquito vectors of this virus are globally distributed in tropical and temperate zones, providing the opportunity for chikungunya virus to continue to expand into new geographic regions. In October 2013, locally acquired cases of chikungunya infection were identified on the Caribbean island of Saint Martin, signaling the arrival of the virus in the Western Hemisphere [6]. As of 16 Dec. 2014, the Centers for Disease Control and Prevention (CDC) has recorded 2,021 U.S. traveler cases in 46 states and Washington, DC; of these imported cases, 1,989 were from the Americas, ten from the Pacific Islands, and eleven from Asia. In 2014, locally transmitted cases occurred in Florida and in Guatemala, Colombia, Venezuela, and Honduras. That year, cases increased dramatically, accounting for over 90% of all new cases occurring in the Americas [9]. Countries in the Americas reporting only imported cases include Argentina, Bolivia, Canada, Chile, Cuba, Peru, and Uruguay. To date, locally acquired chikungunya infections have occurred in more than 40 Western Hemisphere countries and islands (U.S. CDC, PAHO, Jan. 2015). The status of chikungunya virus has moved from anonymity to notoriety and it has now become a major emerging threat on a global scale.

STUDY AREA

Guatemala is a Central American country bordered by Mexico in the north, Belize to the

northeast, El Salvador and Honduras in the south, and the Pacific Ocean and Gulf of Honduras to the west and east, respectively.

Figure 1: Departments in Guatemala. (source: ArcView GIS, author)

Guatemala is the most populated country in Central America and half of its population of over 14 million people are under the age of 19 [10]. The country has experienced significant growth as its population has doubled in the last 25 years and is expected to increase by another 14 million in the next 25 years [10]. This exponential growth will continue to present significant challenges in the realm of infrastructure and public health support given the level of poverty and lack of access to quality basic services in the country.

The port city of Puerto Barrios is located within the Izabal Department on the Amatique Bay, which is on the relatively small eastern coast of Guatemala (Fig. 1). The population in Guatemala is at risk for several significant mosquito-borne diseases including malaria and dengue fever. Malaria is present in the country with a morbidity and mortality index of 3.1 per 1,000; *Plasmodium vivax* is the major malaria parasite found there [11], with most cases occurring in the northern part of the country away from Puerto Barrios. Furthermore, all four serotypes of dengue virus are present in Guatemala with Puerto Barrios in an endemic area. Overall, the burden of vector-borne disease in Guatemala is estimated to be 0.7 disability adjusted life year (DALYs) per 1,000 people per year. As of December 2015, Izabal district where Puerto Barrios health department is located have reported 16,000 – 32,000 cases of

chikungunya since the beginning of 2015 (6), and although there are clinical presentations of the disease in humans, there are few resources for collecting seroprevalence data. There are several military platforms that conduct capacity engagement exercises in Puerto Barrios in which active duty personnel are present on the ground for six months at a time as well as short duration missions to include Continuing Promise, Southern Partnership Station, and New Horizons (U.S. Southern Command). Personnel exposed to chikungunya returning from infected regions present a source of infection to local *Aedes* mosquitoes throughout their range in the United States.

SIGNIFICANCE

Recent massive outbreaks in different parts of the world emphasize the need for further investigation to better understand the underlying factors contributing to the severity and rapid spread of chikungunya.

Proactive field surveillance of adult mosquitoes and disease diagnostics are necessary to better track the presence of chikungunya virus and prioritize vector control measures to reduce the spread of the disease. The presence of chikungunya virus and its distribution is known by clinical diagnosis and seroprevalence data on a country-by-country basis [9]. Understanding the distribution and prevalence of the vector and the virus in combination with prioritized vector control measures may reduce the incidence of disease in local populations at risk.

The disease is still spreading and currently geospatial information and predictive data on where chikungunya virus will next occur is lacking. Adding data points of vectors positive with chikungunya virus to databases such as VectorMap.org will aid researchers in developing predictive disease models as well as anticipate countermeasures in preventing the emergence in naive populations. Susceptible military populations operating in Central America and the Caribbean are vulnerable to exposure to the virus and most of these countries have limited public

health resources available for mosquito surveillance and arbovirus detection. Isolating the virus in the mosquito will provide prevalence data in the mosquito population as well as an understanding of the potential threat to U.S. personnel working in Puerto Barrios, Guatemala.

STUDY OVERVIEW

This research is a characterization study of *Aedes aegypti* and *Aedes albopictus* (if present) to determine the distribution and proportion of mosquitoes in urban and rural settings in Puerto Barrios, Guatemala, and finally to test for the presence of chikungunya virus infected *Aedes* species and the proportion of positive and negative virus results in urban and rural settings to determine which setting the *Aedes* spp. is statistically significant.

The objectives were to:

1) Identify and record field-collected mosquito samples by genus and species (*Aedes* spp. only) and their relative densities from field data documented by Navy Environmental and Preventive Medicine Unit Two (NEPMU-2) personnel.
2) Determine if *Ae. albopictus* is established and identify preserved mosquitoes collected in the NEPMU-2 laboratory.
3) Compare *Aedes* spp. populations in urban and rural settings, test for chikungunya virus-infected mosquitoes and determine the proportion of positive and negative results based on urban and rural cases.

Based on a sample size of n=88 pools of mosquitoes for a 21 day sampling period, 44 samples in urban environment and 44 in rural are needed. Using a 2x2 contingency table (Fig 2.) and Chi-Square test at an $\alpha = 0.05$ level of statistical significance may achieve greater than 80% power if 20% in the urban mosquito samples test positive and 5% for rural samples. Current

information on population chikungunya cases from the Ministry of Public Health (MSPAS) suggests that positive pools will occur [12]. RT-PCR analysis results will be used in a 2X2 table due its greater sensitivity in detecting virus .

	Virus (+)	Virus (-)
Urban		
Rural		

Figure 2. Contingency 2X2 table for Virus detection in *Aedes* mosquitoes.

Proportion of species	*Ae. aegypti*	*Ae. albopictus*
Urban		
Rural		

Figure 3. Contingency 2X2 table for proportion of species.

CHAPTER 2: Literature Review

CHIKUNGUNYA CASES

Since 2004, chikungunya virus has caused millions of cases of disease in the Indian Ocean basin and has emerged in new areas including Europe, the Middle East, and the Pacific region [6]. The mosquito vectors of this virus are globally distributed in tropical and temperate zones, providing the opportunity for chikungunya virus to continue to expand into new geographic regions. In October 2013, locally acquired cases of chikungunya infection were identified on the Caribbean island of Saint Martin, signaling the arrival of the virus in the Western Hemisphere [6]. As of 16 Dec. 2014, the Centers for Disease Control and Prevention (CDC) has recorded 2,021 U.S. traveler cases in 46 states and Washington, DC; of these imported cases, 1,989 were from the Americas, ten from the Pacific Islands, and eleven from Asia. In 2014, locally transmitted cases occurred in Florida and in Guatemala, Colombia, Venezuela, and Honduras, cases increased dramatically, accounting for over 90% of all new cases occurring in the Americas [9]. Countries in the Americas reporting only imported cases include Argentina, Bolivia, Canada, Chile, Cuba, Peru, and Uruguay. To date, locally acquired chikungunya infections have occurred in more than 40 Western Hemisphere countries and islands (US CDC, PAHO, Jan. 2015).

VECTOR COMPETENCE AND DISTRIBUTION

While both *Ae. albopictus* and *Ae. aegypti* are competent vectors of chikungunya virus, each species is reportedly responsible for transmitting different strains of the disease [13]. Outbreaks in the Indian Ocean basin have been attributed to *Ae. albopictus*, while outbreaks in Asia have been associated with *Ae. aegypti* [13]. Currently, it is believed that the strain

circulating in the Caribbean is closer to the Asian genotype [14]. Travel to and from the Caribbean is frequent (the CDC reports approximately 9 million U.S. residents travel to the Caribbean each year), and U.S. forces operating there as well as other chikungunya-endemic regions are susceptible to infection. *Aedes aegypti* is known to occur in the southern United States and is common in urbanized areas throughout the Caribbean and in Central/South America [15].

AEDES (*STEGOMYIA*) *ALBOPICTUS*

The prevalence of *Ae. albopictus* in Guatemala and many of the Central American countries is largely unknown, and this mosquito has not been formally implicated in transmitting chikungunya virus in Guatemala. In the U.S., *Ae. albopictus* may have displaced *Ae. aegypti* [7] throughout much of its original Southeastern U.S. range. *Aedes albopictus* was not recognized in Guatemala [8] and on Hispaniola (in the Dominican Republic) until the early 1990s [2] and it has only recently been reported in Haiti in 2012 [3]. This likely reflects lack of entomological surveillance in most Central/South American and Caribbean countries. It is unclear how both species will coexist in similar ecological niches in these locations and what epidemiological implications this may have on the prevalence of chikungunya transmission. *Aedes albopictus* has demonstrated being a competent vector in numerous other chikungunya virus outbreaks in the Indian Ocean region and Europe. The adaptation of chikungunya virus to *Ae. albopictus* mosquitoes likely contributed to the dramatic spread of chikungunya virus in the Eastern Hemisphere [6]. Evidence of transmission of the chikungunya virus through *Ae. albopictus* in Guatemala may also elucidate the potential emergence for locally transmitted chikungunya virus in the Mid-Atlantic region of the U.S. in which *Ae. albopictus* is ubiquitous among areas of significant human population density.

TRAPPING AND COLLECTION OF MOSQUITOES

The BG-Sentinel™ trap (Biogents AG, Regensburg, Germany, Fig. 4) used in conjunction with the Biogents human odor lure is currently the most effective trap combination yielding greater specificity in collecting *Ae aegypti* and *Ae. albopictus* [16]. The behavior of *Ae. aegypti* is more anthropophilic (predominantly feeds on humans) and using the human odor lure with the BG-Sentinel™ increases the traps' specificity of mosquito species collected [17]. *Aedes albopictus* is known to be more zoophilic (non-discerning blood feeder) that will feed on mammals and birds and may be less strongly attracted to the BG-Sentinel™ trap with the human odor lure [17]. The BG-Sentinel™ trap and human odor lure were specifically designed to collect *Ae. aegypti*, and utilizing this method of mosquito trapping may not reflect the true ratios of *Ae. aegypti* and *Ae. albopictus* when these two species coexist [17]. Studies have suggested that *Ae. albopictus* continues to outcompete *Ae. aegypti* in rural areas while *Ae. aegypti* is outcompeting *Ae. albopictus* in some urban areas in the U.S. such as Florida [18].

Figure 4. BG-Sentinel™ Mosquito trap.

CHAPTER 3: Methodology

RESEARCH GOAL

The goal of this study was to characterize *Ae. aegypti* and *Ae. albopictus* (if found) mosquitoes collected and preserved from a Department of Defense (DoD) mission in Puerto Barrios, Guatemala, to determine relative densities, proportions in comparison of urban and rural settings, and finally determine if the collected mosquitoes were infected with chikungunya virus.

HYPOTHESIS

This research will test the following hypotheses:

1) The mosquito *Ae. albopictus* is established in Guatemala
2) Chikungunya virus is present in *Ae. aegypti* and *Ae. albopictus* (if present in Guatemala).
3) The proportion of chikungunya virus-infected mosquitoes from urban-collected samples will produce higher infection rates than rural-collected samples.

RESEARCH OBJECTIVES

1) Request data and specimens on field-caught adult mosquito collections with locations in Puerto Barrios, Guatemala from cooperative engagement sites where DoD personnel are engaging with Guatemalan military and local Ministry of Health personnel.
2) Examine field samples collected by the Navy Environmental and Preventive Medicine Unit TWO, Norfolk, VA (NEPMU-2) for the presence of *Ae.*

albopictus and *Ae. aegypti* and characterize distribution in Puerto Barrios, Guatemala using urban and rural trap locations.

3) Establish presence of chikungunya virus in mosquito samples from Guatemala by conducting RT-PCR analysis on preserved mosquitoes collected using only mosquito legs to determine disseminated chikungunya virus.

4) Compare rural vs. urban *Aedes* spp. for presence of chikungunya virus and determine statistical significance of any difference using a Chi-Square test.

5) From positive trap pools, determine the minimum infection rate: ([number of positive pools/total specimens tested] x 1000), and compare infection rates for each species of mosquito.

6) Estimate the distribution of *Aedes* spp. mosquitoes in Puerto Barros, Guatemala from NEPMU-2 collected samples. Submit collected species taxonomic data and positive chikungunya results to VectorMap (http://vectormap.si.edu/) in order to generate disease maps, mapped collection data and distribution models for arthropod disease vector species.

VECTOR SURVEILLANCE ACTIVITY IN GUATEMALA

Existing mosquito specimens and trapping data will be obtained from NEPMU-2, Norfolk, Virginia. Global Positioning Systems (GPS) were used by NEPMU-2 to keep records of surveyed areas, trap locations, and other relevant geographical information pertinent to vector populations.

Adult mosquito trapping techniques consisted of BG-Sentinel^{TM} traps baited with lure (Fig. 4), a validated method to trap *Aedes* spp.[16], at preselected locations using a total of four traps. Five urban locations with a single trap placed outdoors, and five rural locations with traps

similarly placed, were monitored; the outdoor traps were set close to the house in an area favored by resting mosquitoes (e.g., under shaded eaves, near doors, next to shrubbery). Surveillance data will be systematically collected (i.e., adult *Aedes* individuals will be counted per trap) to determine relative densities of vectors at particular trap locations. Selected house site locations were based on prior history of dengue or chikungunya infections. No human data or personal identifiable information was collected.

SPECIMEN COLLECTION

Trapping occurred over a 24 hour time interval to ensure uninterrupted trapping during peak mosquito feeding periods which occur during midmorning and early afternoon for daytime-biting *Aedes* mosquitoes [19]. Traps were activated shortly after sunset and allowed to run 24 hours before mosquitoes were removed, at which time trap batteries were replaced and empty collection bags re-set within traps. *Aedes* mosquitoes were identified to species (other mosquitoes collected were identified to genus) by a trained U.S. Navy Environmental Health Officer and Preventive Medicine Technician using morphological characters. Male mosquitoes were discarded and female mosquitoes were data logged and labeled for time, day, and location with writable tape and then returned to NEPMU-2 and placed in the -80 Celsius freezer for subsequent species identification confirmed by a board certified entomologist.

MOSQUITO PRESERVATION AND PROCESSING

Mosquitoes were separated and identified to genera and species (*Aedes* spp. only) by NEPMU-2 personnel using a 10x microscope and the dichotomous key of Rueda (2004). Whole mosquito specimens were preserved in RNALater® (Qiagen, Germantown, MD) by trapping locations in 10ml specimen tubes labeled A-O representing locations and transported to NEPMU-2 for testing. RNALater® is a non-toxic liquid storage reagent that has been used to

preserve mosquito RNA at room temperature up to one month, and indefinite storage time is possible if held at -20°C [20].

Carrier RNA from a 310 µL in five aliquots were prepared as this enhances binding of viral nucleic acids to the QIAamp membrane. Mosquitoes were removed from the RNALater® containing specimen tubes and placed in a clean mortar and pestle. Whole mosquitoes were homogenized in a solution of 560 µL AVL (buffer) and 5.6 µL of RNA AVE until all mosquito parts are visibly indistinguishable. The homogenized solution was extracted into a 1000 µL specimen tube and labeled with the respective sample pool letter A-O. The homogenized solution was allowed to incubate for 10 minutes on the lab table specimen tube rack. Following 10 minutes, the specimen tube was centrifuged for 10 minute and 6Gs then transferred to corresponding letter labeled lysis tube. 560 µL of pure ethanol was added to the lysis tube and centrifuged for one minute at 6Gs. Wash AW1 of 500 µL was added and spun for one minute at 6Gs, the wash discarded and transferred. An additional wash of AW2 500 µL was spun for one minute at 6Gs, discarded, and the wash wand transferred to the lysis tube. Following the wash procedure, a dry spin at 7Gs was required for 10 minutes. Once the dry spin was completed, a 60 µL buffer of AVE was added directly to the lysis filter and allowed to incubate at room temperature for one minute then spun for one minute at 6Gs. This extraction procedure was conducted on all mosquito samples A-O.

An initial spiked sample utilizing the positive control of the RealStar® Chikungunya RT-PCR Kit 1.0 (Altona Diagnostics, Hamburg, Germany) and two *Culex salinarius* Coq. mosquitoes collected at Camp Lejeune, North Carolina were utilized as negative controls. *Culex salinarius* have not been documented to be a competent vector for chikungunya virus. All prepared samples were placed in a 96 well plate and a master mix of 126 µL provided six

reactions in order to compensate for pipetting method errors. The initial well plate samples consisted of a duplicate spiked sample (positive control) and duplicate mosquito samples of *Cx. salinarius.* Prepared samples A-O were placed in duplicates in the well plate and then processed utilizing Applied Biosystems 7500 Fast DX RT-PCR Instrument.

CHAPTER 4: Results

COLLECTION OUTCOME

A total of 106 mosquitoes were trapped and collected at randomly assigned areas in urban and rural settings in and around Puerto Barrios. All mosquito species were collected and identified for data logging purposes to include *Anopheles* spp., *Culex* spp., *and Aedes* spp. *Aedes aegypti* and *Ae. albopictus* were isolated and persevered for further analysis (Fig. 5). Only female mosquitoes were collected and identified, no male mosquitoes were included among the

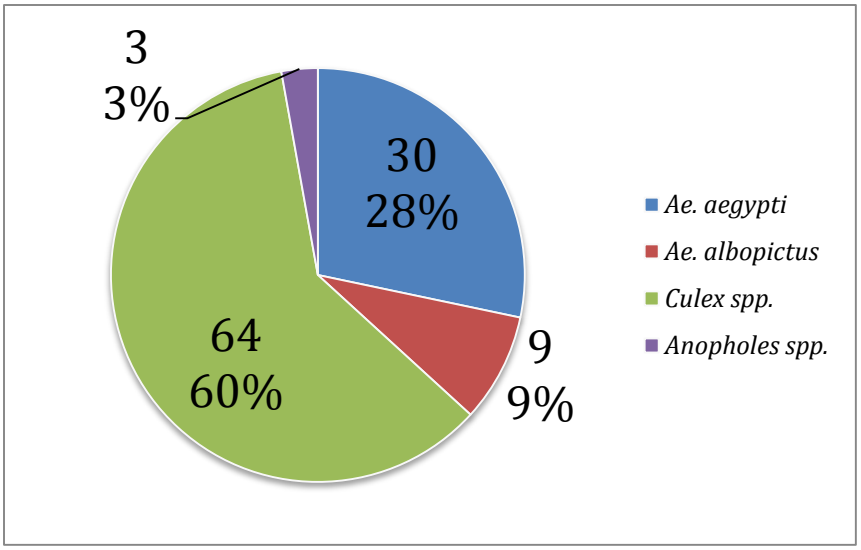

Figure 5. Total mosquitoes collected from BG-Sentinel™ traps and proportion of species abundance, Puerto Barrios, Guatemala.

106 mosquitoes tested. Trap locations were recorded using a global positioning system (GPS) device and mosquitoes were pooled from one to nine *Aedes* spp. per pool. Mosquito pools were separated by species into *Ae. aegypti* and *Ae. albopictus* from each collection point and date. Only pooled and homogenized *Aedes* spp. specimens were examined for laboratory confirmation for the presence of virus. The proportion of collected mosquitoes were 68% *Culex* spp., 37%

Aedes spp., and 2% *Anopheles* spp. Of the 37% *Aedes* spp. collected, a small proportion were *Aedes albopictus* (7%) and the remaining were *Aedes aegypti*. All mosquitoes were field identified by trained personnel and formally recorded by location using a GPS device. A staff entomologist at NEMPU-2 provided identification of the retuned specimens using a dichotomous key.

Figure 6. *Aedes albopictus* identified to species by a median-longitudinal white stripe and photographed under 10x magnification using a dissecting microscope.

The voucher or proof we collected and tested, *Ae. aegypti* and *Ae. albopictus* specimens, consisted of identification from a trained staff entomologist and a photograph of the collected specimens under the laboratory stereoscope (Fig. 6).

PROPORTION OF *AEDES* SPECIES

The observed abundance of *Aedes* spp. was recorded individually by locations in the rural and urban environment. *Aedes aegypti* was found to be predominately established in both the

urban (14, 74%) and rural (16, 80%) settings for a total of 30 specimens collected. A significantly small proportion of *Aedes albopictus* was observed in both the urban (5, 26%) and rural (4, 20%) setting for a total of nine specimens collected.

DENSITY OF MOSQUITOES COLLECTED

All trapping locations were recorded with corresponding GPS coordinates in order to establish the abundance of *Aedes* spp. in both categories of urban and rural environments. The BG Sentinel™ trap and BG-Lure is effective in trapping *Aedes* spp. as well as *Culex quinquefasciatus* [16]. A significant proportion of mosquitoes collected

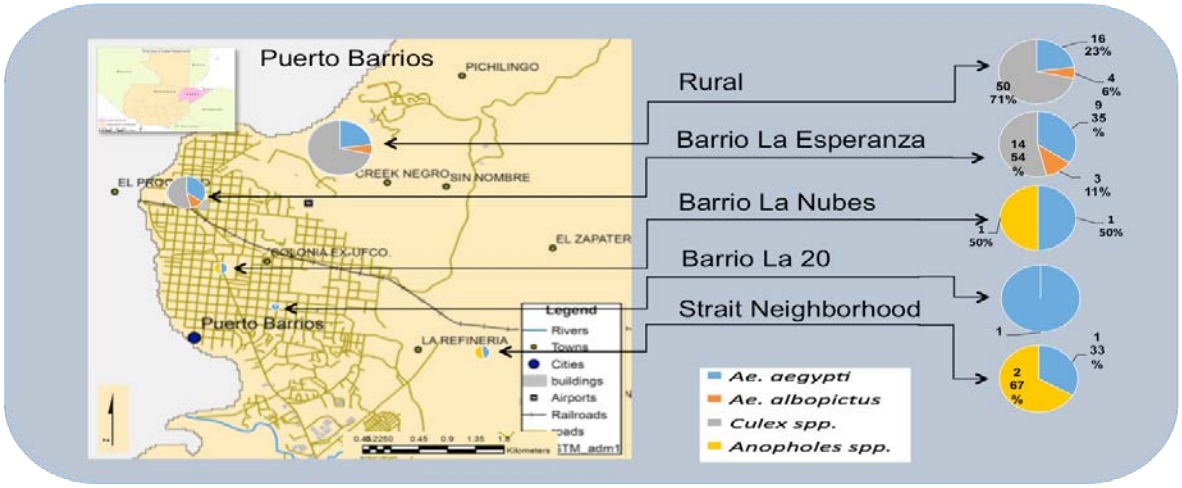

Figure 7. Mosquito species population density by sampled locations.

using this trap and lure were recorded as *Culex* spp. (60%). This mosquito genus was not keyed to species and was discarded after collection recording.

An abundance of *Culex* spp. was observed in rural areas characterized with more vegetation such as trees and bushes than nearby urban areas. The location of Barrio La Esperanza is considered urban and it is located along the coast of Puerto Barrios even though the community has a lower population density than other urban-identified trapping locations. The

rural location consisting of an area estimated to be 1,000m^2 had four trapping sites within the designated area to include the DoD personnel contingency supporting the vector surveillance data collection effort. *Culex* spp. (71%) was observed in significant proportions over *Aedes* spp. (29%) collected in an estimated 3:1 ratio of *Culex* spp. to *Aedes* spp. This observation is consistent with the BG Sentinel™ trap and lure collection results in high vegetation areas selected for trapping and the trap's effectiveness in *Culex* spp. collection in rural forested environments [16]. *Anopheles* spp. was observed in small numbers making up 3% of the total mosquitoes collected.

RT-PCR RESULTS OF *AEDES* SPECIES POOLS

Fifteen *Aedes* spp. pools identified by letters A through O representing sampled locations (Fig. 8) and samples were placed in pairs on the 96 well plate and analyzed for chikungunya virus using the RealStar® Chikungunya RT-PCR kit version 1.0. Two *Cx. salinarius* mosquitoes collected in Camp Lejeune North Carolina were utilized as negative controls. The positive control was a spiked sample of the positive sample media contained in the RealStar® Chikungunya RT-PCR kit version 1.0 (Altona Diagnostics, Hamburg, Germany).

Ten pools (A/B/C/E/F/L/H/I/N/M) represented the *Aedes aegypti*-collected samples and five pools (D/G/K/O/J) represented *Aedes albopictus*-collected samples (Fig. 8). Thirty-four of the 96 wells were occupied in this analysis from the collected mosquito samples. Negative chikungunya virus amplification was observed in negative control as well as negative virus amplification on all mosquito pools A through O. Chikungunya virus amplification was found in the positive control/spiked sample as expected.

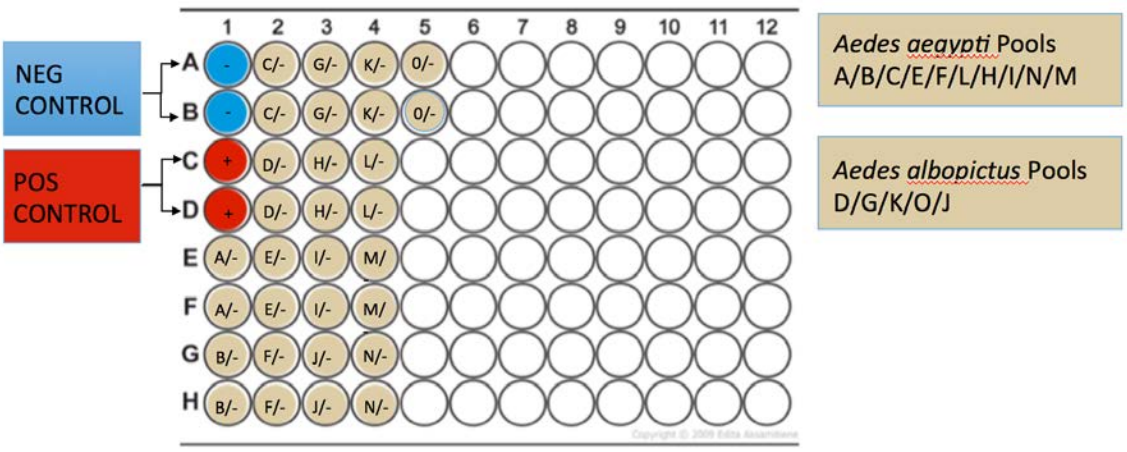

Figure 8. RT-PCR results of 96 well plate.

CHAPTER 5: Discussion

STUDY OUTCOME

This research tested the following hypotheses:

1) The mosquito *Aedes albopictus* is established in Puerto Barrios, Guatemala.
2) Chikungunya virus is present in *Aedes aegypti* and *Aedes albopictus* (if present in Puerto Barrios).
3) The proportion of chikungunya virus-infected mosquitoes from urban collected samples will produce higher infection rates than rural collected samples.

Hypotheses #1 and the specific aims to support the characterization of *Ae. albopictus* were achieved although the sample size and density of the collected species did not yield significance in the determination of whether this mosquito is established in either rural or urban settings. Hypothesis #2 and #3 supported the analysis and presence of chikungunya-infected *Aedes* mosquitoes as well as the novel findings in *Ae. albopictus* in which research has suggested to be a competent vector of the virus. Hypothesis #2 and #3 could not be proved or disproved due to the small number of mosquitoes collected; however, useful information was gained about the collection and testing of mosquitoes for chikungunya virus that will be helpful in future research endeavors.

MOSQUITO PRESERVATION WITHOUT COLD STORAGE AVAILABILITY

Virologic detection of chikungunya and similar RNA viruses rely on identifying viral RNA through RT-PCR methods in the laboratory. Mosquito specimens are traditionally collected and preserved utilizing dry ice (-70^{o}F) in areas without immediate laboratory diagnostics in order to minimize viral degradation and maximize detection.

Puerto Barrios does not have access to dry ice and shipping in the region. The closest shipping facility is located in Guatemala City, a six hour car drive. Many Central American and Caribbean countries lack the resources necessary to maintain a cold chain as the mosquito samples return to laboratories for virus testing.

Prior to conducting this research, we consulted with USAMRIID medical entomologist, Dr. Michael Turell on determining methods of preserving mosquito specimens without cold chain storage availability as well as how suboptimal holding temperatures affect the ability to detect viruses in pools of chikungunya-infected mosquitoes. His study demonstrated chikungunya viral detection was possible after 14 days at room temperature without the use of RNALater® [21]. Chikungunya virus detection is possible for two weeks under sub-optimal holding temperatures [21].

Protocols in field collection of mosquitoes in absence of available cold chain methods also suggest using RNALater® to reduce viral degradation during transportation [20]. Recent studies have also demonstrated the persistence of chikungunya viral RNA in experimentally inoculated *Ae.aegypti* mosquitoes stored for prolonged periods at room temperature to 12 weeks while applied to an adhesive tape for storage and transportation [22].

This study collected mosquitoes without the benefit of a cold chain and relied on the short transportation times (5 days via military ship transport) and room temperature storage conditions from the collection sites in Puerto Barrios. Mosquito pools were frozen at 0°C in order to kill for identification and segregation purposes. They were then placed whole-body in sterile collection tubes containing RNALater®. Although 15 pools of *Aedes* spp. mosquitoes were negative with respect to chikungunya viral RNA, the short duration period of transportation and use of RNAlater® suggest that mosquito pools held at longer transportation periods from

endemic areas would validate the legitimacy of our negative chikungunya virus results due to the persistence of chikungunya virus in *Aedes* spp. using RT-PCR detection methods.

DENSITY AND PROPORTION OF MOSQUITOES

The abundance of mosquitoes observed per trap during the trapping period was not expected to be low based on the seasonal time frame, geographical location, and trapping effectiveness of the BG-Sentinel™ for *Aedes* spp [17]. There currently is no trapping data to base an estimation of the amount of mosquitoes expected to be collected during a trapping period in Puerto Barrios; however, the trapping schedule was aligned with the end of the rainy season to optimize daily *Aedes* collections. The rainy season usually brings periodic tropical showers in the late afternoons in this region providing a diversity of standing water-breeding opportunities favored by *Aedes* mosquitoes breeding in urban environments. Despite these favorable conditions, the highest count of collected *Ae. aegypt*i was nine mosquitoes obtained from a single trapping pool/location while one mosquito was collected in the lowest pool with an overall average of three mosquitoes per pool.

SPECIES VOUCHER

In order to provide a suitable voucher or evidential findings of *Ae. albopictus,* public health personnel were trained in the field identification of *Aedes* spp. using the dichotomous key of Rueda (2004) and stereoscopes to visually confirm that *Ae. albopictus* was collected. Guatemalan field entomologists were unavailable to positively identify collected mosquitoes, therefore, previously trained preventive medicine personnel identified the mosquito species. The final species determination was performed at the NEMPU-2 laboratory. The mechanical nature of the BG Sentinel^{TM} trap and the run time of the trap's fan may have forced the removal of differentiating characteristics dorsally on the thorax such as the median-longitudinal white stripe

that indicates in the dichotomous key of Rueda (2004) that the specimen is *Ae. albopictus.* Transportation of collection tubes with the whole mosquitoes immersed in RNALater® may have dislodged scales and other morphological characteristics necessary to classify and provide a valid voucher of collected *Aedes* mosquitoes. The mosquitoes were not cryogenically preserved in -80°C and this project utilized intermittent cold storage and RNALater® as a primary preservation protocol due to the austere sampling and collection conditions. The mosquitoes returned to the lab for confirmatory identification were in poor condition making final determination of the species difficult. Therefore, we utilized the trained preventive medicine personnel in mosquito identification data of *Aedes* species as a confirmation of collected and properly identified *Ae. aegypti* and *Ae. albopictus.*

AEDES ALBOPICTUS CHARACTERIZATION

It is undetermined if *Ae. albopictus* is established in the community or has out-competed *Ae. aegypti* in the region due to the limited sample size and sample duration period. The observations of the proportions and distribution of *Ae. aegypti* during the collection period suggest that *Ae. aegypti* remains established in Puerto Barrios, Guatemala. Characterization of *Ae. albopictus* in this research proved that the mosquito is present in both urban and rural settings yet the abundance and proportion observed indicates that the species is present but may not yet to be well established in this region. The trapping distribution in urban environments was limited to four locations ranging from one to two kilometers apart while the rural designated area encompassed samples points 100 – 500 meters apart all in a confined cluster. The field collectors were unable to place traps outside of their assigned bivouac location due to force protection restrictions and the Ministry of Public Health (MSPAS) personnel were also limited in access to daily transportation.

DISSEMINATED VS. NON-DISSEMINATED VIRUS

Chikungunya virus in both *Aedes aegypti* and *Aedes albopictus* has a short minimal extrinsic incubation period of only two days during which the virus replicates to high titers. The mechanism of arbovirus infection is suspected to disseminate from the midgut to secondary tissues and cross barriers between the hemocoel and salivary glands thus transmitting the virus through the saliva [23].

The minimum infection rate (MIR) formula is calculated: [number of positive pools / total specimens tested] x 1000. The calculation represents a single species or species group collected over a time period and geographic area specific to a formally designed surveillance program. The MIR uses the assumption that a positive pool contains only one infected mosquito, an assumption that may be invalid when infection rates are high [24]. The smallest sample size of *Aedes* mosquitoes was a pool of one mosquito trapped at designated urban and rural locations. In order to determine if the collected mosquito in a pool was infected and that chikungunya virus was disseminated throughout the mosquito including the salivary glands making the mosquito infective, mosquito legs need to be processed separately from the head using RT-PCR with the remaining body parts discarded. The mechanism to homogenize one mosquito in a sample pool with RNA extraction salts requires a mortar and pestle. Liquids and the mechanical crushing of the mosquito specimen's legs were ineffective using this method due to the surface tension of the liquid as well as the limited movement of the mortar and pestle; mosquito legs were unable to be mechanically crushed which could of resulted in the loss of RNA for detection. Using whole mosquitoes and crushing the specimens in the available mortar and pestle was effective, however; blood meal contamination in a pool would provide false positives of infected mosquitoes and an inaccurate MIR calculation.

PROJECT LIMITATIONS

Sample Size of Mosquito Pools Collected

The proposed sample plan required a 21-day sampling period in which 88 sample pools would be collected, 44 sample pools from rural sites and 44 sample pools from urban sites. Using Chi-Square test at an $\alpha = 0.05$ level of statistical significance could have achieved greater than 80% power if 20% in the urban mosquito samples tested positive for chikungunya virus and 5% tested positive in rural samples. Reports from the local Ministry of Public Health (MSPAS) stated that an emergency chikungunya outbreak in the region of Izabal was occurring and they subsequently conducted an aggressive adulticiding campaign. The majority of control efforts focused on thermal fogging with pyrethroid insecticides, typically resmethrin. The MSPAS vector control provides adulticiding for the community and it is not privately contracted. Additionally, the presence of U.S. Forces may have added pressure on the local MOH to provide aggressive fogging campaigns as a mission engagement request of the State Department and Military Group/Military attaché (MILGRP) for military activities occurring in this area. There was no recorded long-term rain event during this time, only intermittent rainfall that is typical of the season. Both the fogging campaign and the limited rainfall may explain why such low numbers of mosquitoes were collected during the 24hr run cycle. No trapping equipment malfunctions were noted such as battery or mechanical failures. The result of collections yielded only 15 pools, eight pools of *Aedes* spp. in the rural environment and seven pools of *Aedes* spp. in the urban environment, out of the proposed 88 sample pools required.

Temporal Limitations

Duration of the sample period was also limited due to several unexpected local restrictions. The DoD public health contingent was available for a five-week mission from the end of August though the beginning of October. Coordination with Ministry of Public Health and

Mr. Hector Soriano was arranged before their arrival, however; Mr. Soriano was unexpectedly unavailable and instead had requirements in another district to provide vector control services. Furthermore, the election period for the governor's seat of Izabal and mayoral seat of Puerto Barrios occurred during trapping efforts, curtailing the availability of MOH entomologists to perform trapping activities. Reports of an anti-American sentiment in Puerto Barrios further restricted movement outside the camp and the public health personnel could not contact or coordinate with Mr. Soriano until the restriction was lifted. Out of the proposed 21-day sample period, MSPAS personnel supported only six days of sampling, greatly reducing the variability of the collection sites. The aggressive adulticiding campaign may have reduced the collection abundance at all or most of the trapping locations. The trapping of mosquitoes in the rural location did yield larger counts of *Aedes* spp. in comparison to the urban locations; however, rural traps were setup for three more days than the urban traps due to the daily access of the site location of the mission personnel.

LABORATORY PROTOCOL DEVELOPMENT FOR RNA ARBOVIRUS DETECTION

Navy Environmental Health and Preventive Medicine Unit TWO microbiology laboratory had not performed a mosquito virus specimen extraction for RT-PCR analysis prior to this research project. Mosquito specimens are sent to DoD laboratories such as US Army Public Health Command, Aberdeen Proving Ground, Maryland, and to the U.S. Air Force School of Aerospace Medicine, Wright-Patterson Air Force Base, Ohio. They are the only current DoD-established laboratories providing detection analysis of infected mosquito populations for all DoD activities and installations. The Navy and Marine Corps Public Health Center is a program and policy command and detection and analysis operations are delegated to the regional field activities (NEMPUs). Those regional field activities support specific areas of responsibility

(AOR). For example, NEMPU-2 provides scientific and technical expertise as well as preventive medicine resources for health threats in support of the U.S. Eastern seaboard and the U.S. Southern Command that consist of host nation partners from Belize to South America. The mosquito homogenization and RNA extraction protocol for RT-PCR analysis preparation used in this research protocol could be used by the NEPMUs to increase their capability to conduct detection analysis of collected mosquitoes in their areas of responsibility. This capability may become critical in the face of new and emerging threats such as Zika virus in the Americas [25]. If mosquitoes are collected in austere environments and have limited cold storage mechanisms, using the RNALater® method of mosquito preservation for subsequent detection analysis is described in the methods section.

CAPACITY BUILDING AND GLOBAL HEALTH ENGAGEMENTS

Central American countries face major challenges in the control of chikungunya, dengue, and Zika viruses. To date, little research has investigated how to develop capacity and target response efforts in regards to vector control or formally establish an integrated community-based vector surveillance program in which key indicators and partnership would signal intervention. The current demand signals for vector control intervention is clinically-based reporting from local health clinics to the local ministry of public health [12].

With the lack of vector surveillance resources, communities suffering from mosquito-borne diseases such as chikungunya, dengue, and Zika, experience a lack of health workers assigned to conduct vector control services. These communities are rapidly expending resources to combat disease cases. Currently, Puerto Barrios, Guatemala does not conduct mosquito trapping surveillance nor does the city possess the ability to purchase trapping devices such as a BG-Sentinel™ trap and its accessories; however, as part of this project Puerto Barrios now

maintains four BG-Sentinel™ traps and accessories as well as having received training on how to field traps within the community. Communication with Mr. Hector Soriano confirmed yearly missions that DoD continue to engage with military-to-military partners in Puerto Barrios providing military health professionals such as entomologists, environmental health specialists, and other preventive medicine personnel long-term sustainability in vector surveillance activities and information sharing. Furthermore, rapid detection assays are available for field detection [26] of chikungunya virus that could provide MIRs in the community and target areas based on those attack rates from mosquito-collected pools utilizing an established vector surveillance program. Puerto Barrios does not maintain a laboratory for detection of human viral cases or infected mosquitoes. This incremental capacity building engagement may assist in establishing an integrated community-based vector surveillance program and assist in determining disease prevalence occurring in local communities.

CHAPTER 6: Conclusion

This is the first study to attempt to determine the presence of chikungunya-infected mosquitoes in a Puerto Barrios, Guatemala, and required a multiagency and multidisciplinary approach to ensure mosquito samples were collected and preserved for further analysis from an austere environment with limited access to field collection sites. Several Central American and South American countries lack resources to determine the infection rates of emerging arboviruses in either naive populations or endemic mosquitoes. Several reports from sentinel health monitoring agencies list only clinical diagnoses and/or seroprevalence data of disease and have not demonstrated the abundance or competence of the vectors associated with chikungunya. Guatemala lacks vector population data due to lack of *Aedes* species-specific trapping methods and equipment [27]. Other surveillance tools have not been effective to provide enough specificity of targeted mosquito species such as light traps baited with CO_2 to use as a lure due to a lack of dry ice availability in austere areas. Vector management personnel rely on community-based surveillance such as health clinics reporting cases of chikungunya and dengue to respond with vector control efforts. The majority of countries in Central America do not practice routine mosquito surveillance and combat arbovirus outbreaks through reactionary methods such as adulticiding campaigns (fogging) as seen during this study. This study attempted to determine chikungunya virus infected *Aedes* mosquito populations near reported human cases from the MSPAS epidemiologic tracking at local health clinics for Puerto Barrios and was unable to detect the virus in field-collected mosquitoes because of limited collection days coupled with aggressive adulticiding/fogging campaigns. Lack of vector surveillance resources, especially for *Aedes* species collection, and available laboratory diagnostics prevents areas like Puerto Barrios from determining the prevalence of the disease in vector populations to provide targeted control

interventions and reduce the cycle of infection. Collection of *Ae. albopictus* provides useful information for vector tracking in the Central American region, where limited species collection data and collection activities have occurred, especially in areas south of Belize. Detection of virus in infected mosquitoes to determine vector competency and associated virus transmission cycles has not routinely occurred in this region.

A greater understanding of where, when and at what densities *Aedes* mosquitoes occur in Central America and surveillance for arbovirus-infected mosquitoes will improve targeted prevention strategies in regions where disease is endemic and curtail introduction to new areas. Country regions such as Puerto Barrios currently lack the ability to quickly detect arboviral infections in endemic communities. Surveillance tools and training, especially for daytime biters such as *Ae. aegypti* and *Ae. albopictus*, will allow for a targeted vector control approach to maximize the efficacy of pesticide applications, reduce associated costs and resources used, and improve awareness of vector abundance in Central America. These methods could also lead to improved surveillance and be used as a model for improving this capacity in other developing regions where vulnerable populations are impacted by emerging arboviruses.

FUTURE RESEARCH

The methodology involved in trapping field-caught *Aedes* mosquitoes in austere environments with limited cold storage provisions provides a method for retuning specimens to DoD laboratories for analysis and establishes protocols for RNA extraction from mosquito specimens. Multicounty approaches to surveillance and detection could provide an ecological niche map of vectors and track or model the emergence of virus into other territories. Furthermore, discovering other species that are competent vectors of chikungunya virus could elucidate the potential risk of emergence in areas where these established species may also be a

competent vector for transmission. Finding chikungunya-infected *Ae. albopictus* in the Central American and Caribbean regions could potentially signal the emergence of the virus further north than the subtropics, well into the Mid-Atlantic region of the U.S. in which established vector populations already exist (e.g., *Ae. albopictus*). Further research is needed both spatially and temporally on vectors associated with dengue, chikungunya, and Zika viruses as the emergence of arboviruses continues.

DISCLAIMER

The views expressed in this article are those of the authors and do not reflect the official policies or positions of the Uniformed Services University of the Health Sciences, Department of the Navy, Department of the Army, Department of Defense, or the U.S. Government.

APPENDIX A: DATA TABLE OF COLLECTED MOSQUITOES

Code (Trap)	GPS- (MGRS)	Date/Time	Urban	Rural	Species Count	Ae. Albopictus	Ae. Aegypti	Anopheles	Culex	vials	Notes
1003	16P CC 29525 39239 (Marleny Coloy)	9/25/2015 (1800-0900)	X		4	2	2			D,E	
1004	16P CC 29521 39231 (Barrio la Esperanza)	9/25/2015 (1800-0900)	X		26	3	9		14	G,F	
1002	16P CC 29518 39246 (Barrio Las Nubes)	9/26/2015 (0900-1800)	X		2	0	1	1		A	
1003	16P CC 29609 38573 (Barrio la 20)	9/26/2015 (0900-1800)	X		1	0	1			B	
1003	16P CC 32534 38391 (Strait Nieghborhood)	9/27/2015 (0900-1800)	X		3	0	1	2		C	
1004	16P CC 29209 41008 (Rinconcito Nieghborhood)	9/27/2015 (0900-1800)	X		0	0	0				
1002	16P CC 28168 40425 (Barrio el Centro)	9/27/2015 (0900-1800)	X		0	0	0				
1005											Rural areas had been fogged once a week since JHSV's arrival to GTM
	16P CC 30647 41234 (BAS)	9/25/2015(0730-1830)		X	0	0	0				
	16P CC 30647 41234 (BAS)	9/27/2015 (0730-1830)		X	0	0	0				
	16P CC 30647 41234 (BAS)	9/28/2015 (0515-1800)		X	0	0	0				
	16P CC 30647 41234 (BAS)	9/29/2015 (0800-1830)		X	0	0	0				
	16P CC 30647 41234 (BAS)	9/30/2015 (0515-1840)		X	3	1	2			K,L	
	16P CC 30647 41234 (BAS)	10/01/2015 (0515-1100)		X	1	1	0			O	
1006	16P CC 30802 41421 (Conf Rm)	9/28/2015 (0800-1800)		X	1	0	1			H	
	16P CC 30802 41421 (Conf Rm)	9/29/2015 (1200-1200)		X	33	0	8		25	I	
	16P CC 30802 41421 (Conf Rm)	9/30/2015 (1200-1100)		X	12	0	4		8	N	
1007	16P CC 30669 41189 (The Box)	9/29/2015 (1200-1200)		X	3	0	0	0	3		
	16P CC 30669 41189 (The Box)	9/30/2015 (1200-1100)		X	3	0	0		3		
1008	16P CC 30911 41534 (Berthing)	9/29/2015 (1200-1200)		X	6	2	0		4	J	
	16P CC 30911 41534 (Berthing)	9/30/2015 (1200-1100)		X	8	0	1		7	M	

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