Improving *Aedes* Mosquito Surveillance and La Crosse Virus Screening in Eastern Tennessee

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ABSTRACT

La Crosse virus (LACV), transmitted by infected *Aedes triseriatus*, *Ae. albopictus*, and *Ae. japonicus* mosquitoes is the leading cause of pediatric arboviral encephalitis. Severe cases of LAC encephalitis occur in individuals 16-years-old or younger and may cause permanent neurological damage or fatality. No vaccines exist making mosquito control and disease prevention crucial to public health. Effective screening and surveillance practices are key components to these goals. While a number of standard mosquito surveillance methods exist, continuous testing and improved understanding of vector biology to determine the best ways to implement these methods is important. Additionally, the current standard for screening LACV, RT-PCR, is time consuming, expensive, and inaccessible by many laboratories. I hypothesized that different LACV vectors would be active at different times (objective 1) and that a more efficient molecular method for virus detection can be developed (objective 2). For objective 1, I collected mosquitoes from 19 sites around Knox County from June-September in 2015 using traps previously found to be effective for monitoring LACV vectors. Nets were changed twice a day during "work" or "off-work" hours (9:00-17:00 or 17:00-9:00). Mosquitoes were identified and trap and time of day recorded. A total of 1,223 *Aedes albopictus*, 49 *Ae. japonicus*, and 90 *Ae. triseriatus* were collected. Significantly more LACV vectors were collected from 17:00-9:00. For objective 2, using a positive control, a reverse transcriptase loop mediated isothermal amplification (RT-LAMP) method of virus detection was developed and a dilution series was conducted to compare the developed assay to the standard. Both assays were found equally effective at detecting LACV, but the RT-LAMP is preferable for cost effectiveness and reduced

detection time. This thesis provides research laboratories, health departments, and citizens with important vector surveillance information and an accurate and inexpensive method of screening for the virus. Surveillance information will make it easier for mosquito control districts to effectively monitor vectors. The diagnostic assay can be used in field-lab settings and will provide accurate results in a shorter time than with traditional methods. Together, the increased efficiency in vector surveillance and virus detection provide rapid and accurate results for low cost.

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1. INTRODUCTION

Background

A disproportionate number of vector-borne diseases occur in low-income areas and developing nations (Reiter et al. 2003). The introduction of non-native vectors, including *Aedes aegypti*, *Aedes japonicus* (Peyton et al. 1999), and *Aedes albopictus* (Benedict et al. 2007) has been instrumental in the movement and increased prevalence of many of these pathogens in parts of the United States. Dengue fever has become established in southern parts of Florida and Texas (Beaumier et al. 2014) and Chikungunya virus is rapidly becoming a worldwide threat (Renault et al. 2007; Gibney et al. 2011; Benelli and Mehlhorn 2016). Factors such as increased urbanization, climate change, global air travel, and sea trade play a major role in the movement and introduction of associated vectors (Hopp J.M 2001; Charrel et al. 2007).

The movement and dispersal of different mosquito vectors and, with them, a variety of dangerous pathogens, is a major global public health concern (Gubler 1998). Malaria contributed to over 600 thousand deaths in 2010 alone (Hemingway 2014). While the United States was successful in eradicating malaria, the nation still faces threats from emerging diseases like Dengue fever (Morens and Fauci 2008), West Nile virus (Lanciotti et al. 1999), and re-emerging native vector-borne disease, like La Crosse and Eastern Equine encephalitis (Feemster 1938; Hollidge et al. 2010). Unfortunately, due to limited funding combined with high diagnostic costs associated with reagents and labor, the true severity and prevalence of many of these diseases are not well understood. Thus, it is necessary to increase our understanding of mosquito behavior to improve mosquito control and disease prevention efforts.

Understanding activity patterns of important vectors is paramount to improving mosquito control efforts (Bonds 2012). Avoiding mosquitoes becomes crucial for individual health since mosquito control is often the only option for preventing vector-borne disease as there is no cure for many of these diseases. While climate has an effect on mosquito behavior and abundance, so do cultural environmental factors presented. Despite the presence of both vector and pathogen, people in Texas are less susceptible to Dengue virus than people in parts of Mexico, due to cultural and economic differences such as air conditioning and availability of window screens (Beaumier et al. 2014). *Aedes albopictus*, a vector of a number of arboviruses, is known to quest for hosts during the day (Rightor et al. 1987; Robertson 1988), while *Culex pipiens*, a known vector of West Nile virus, quests at night. This information allows control technicians and citizens to more accurately monitor and control vectors, as well as protect themselves from bites. The differences in behavior between vectors highlight the importance of targeted mosquito control and disease prevention efforts.

Importance of La Crosse virus

One particularly important arbovirus in the United States is La Crosse virus (LACV), first isolated from the brain of a four-year-old girl who died of encephalitis in La Crosse, Wisconsin in the 1960's. La Crosse virus is a single stranded, segmented, spherical, enveloped RNA virus in the California (CAL) serogroup, family *Bunyaviridae* and genus *Orthobunyavirus* (Elliott 1990). While the native mosquito, *Aedes triseriatus*, is the primary vector, it is likely the virus can also be transmitted by two secondary introduced vectors, *Ae. albopictus* (Gerhardt et al.

2001) and *Aedes japonicus* (Harris et al. 2015). The virus is maintained in a zoonotic cycle involving *Aedes* mosquitoes and the reservoir hosts, eastern gray squirrels (*Sciurus carolinensis*) and eastern chipmunks (*Tamias striatus*) (Moulton and Thompson 1971). LACV can also be transmitted transovarially from the female *Ae. triseriatus* mosquito to her progeny (Watts et al. 1972) and horizontally between males and females during mating (Thompson and Beaty, 1978). Humans are a dead end host that do not support enough virus in the blood stream to allow uninfected mosquitoes to acquire the virus (Erwin et al. 2002). Symptoms of the disease include headache, nausea, fever, or fatigue, or in severe cases neurological symptoms and seizures. The severe disease, characterized by encephalitis or meningitis, occurs most often in children under 16-years-old and is more common in males than females (McJunkin et al. 2001).

Approximately 80 to 100 cases of LACV are diagnosed each year in the United States and La Crosse encephalitis is the number one cause of arboviral encephalitis in children. Originally identified in La Crosse Wisconsin, it later moved through the Midwest and has established in the Appalachian region (Haddow and Odoi 2009). For a span of 33 years, only nine cases were confirmed in Tennessee until 1997 when there was a cluster of 10 cases of LACV confirmed in the state (Jones et al. 1999). Since then the number of La Crosse encephalitis (LACE) cases in eastern Tennessee has continued to increase (Haddow and Odoi 2009). There was even a fatal case in Union County, Tennessee in 2012 that sparked an in-depth vector ecology study (Trout Fryxell et al. 2015). However, the disease is often misdiagnosed (McJunkin et al. 2001), delaying treatment and contributing to potentially long-term neurological damage or even fatalities. For these reasons, improved diagnostic methods are becoming increasingly important.

Importance of La Crosse virus Vectors

Of the three known LACV vectors, (*Ae. triseriatus*, *Ae. albopictus*, and *Ae. japonicus*) *Ae. triseriatus* is the only vector native to the United States (Watts et al. 1972). It is a Nearctic, container-dwelling mosquito with a distribution that includes southern Florida, parts of Canada, and parts of the western United States like Utah, and Idaho. It was first identified as a competent vector of LACV between 1964 and 1968 (Watts et al. 1972). It primarily feeds on chipmunks and gray squirrels, which are also the amplifying hosts for LACV (Mather and DeFoliart 1983). Bloodmeals taken from these amplifying hosts have been associated with high fecundity in the *Ae. triseriatus* mosquito (Mather and DeFoliart 1983) making it an especially important factor in the continuing presence of LACV. This mosquito prefers to oviposit in treeholes, small, dark-colored containers of standing water with rough surfaces, and in the presence of dead leaf material (Wilton, 1968) typical of forested habitats. The likelihood of *Ae. triseriatus* females biting humans is increased by movement of people into these forested areas and the increase of artificial containers suitable for larval growth and development.

The species *Ae. albopictus* is a competent vector of many arboviruses that originate in South-East Asia and within two decades has established in Europe, the Middle East, North and South America, and the Caribbean (Gratz 2004). In the United States, *Ae. albopictus* was first discovered in Texas around 1980 in tires (Sprenger and Wuithiranyagool 1986) and its range now includes areas as far north as New York, New Jersey and Pennsylvania (Rochlin et al. 2013). This is a diurnal species that aggressively quests for a variety of mammalian hosts, including humans, dogs, small rodents, and even some reptiles (Niebylski et al. 1994). It has the

ability to transmit a wide variety of pathogens, including Dengue and Chikungunya virus (Benedict et al. 2007) and was found to be a competent secondary vector of LACV (Gerhardt et al. 2001). This is a desiccant-tolerant tree-hole and container-dwelling species that has been moving rapidly across the globe.

Aedes japonicus, a less common secondary vector, was also introduced from Asia and has since spread to Europe and much of North America, including 33 states in United States (Kampen and Werner 2014). It was first discovered in New Jersey in 1998, possibly also through the importation of tires (Peyton et al. 1999). This species also oviposits in treeholes as well as rock pools and artificial containers (Peyton et al. 1999) and feeds primarily on mammals like deer (*Odocoileus* spp.) and horses, as well as humans and some smaller mammals (Molaei 2009). Little is known about its temporal activity, but studies on the physiology of the eyes suggests this species is crepuscular (Land et al. 1999).

Prevention of LACV is possible with control of the vectors and minimizing mosquito contact. Since humans are a dead-end host and LACV can only be transmitted via mosquito bites. Home window screens exclude mosquitoes from the structure and adequate air conditioning allows for closed windows without sacrificing human comfort. Outdoors, effective insect repellents, especially those containing DEET can be used. Removing standing water from yards, cleaning gutters, and maintaining yards can prevent mosquitoes from ovipositing eggs and increasing in numbers (CDC 2009).

Mosquito Surveillance

Traps designed to attract and collect mosquitoes at different life stages are crucial to accurate surveillance and eventual control of vectors and pathogens. Increased understanding of vector species is necessary for designing the most effective and efficient traps. We must evaluate the surrounding environment (vegetation, urban or rural setting, host species) and temporal activity of vectors. Timing of trapping different vector species must take into account when they are most likely to be active.

Questing traps are designed to collect mosquitoes seeking a blood meal. These traps generally rely on CO_2 or chemical lures and include CDC miniature light traps, BG sentinel traps, and Fay Prince traps (Kline 2006; Krockel et al. 2006). Although ethically questionable in high-risk areas, human landing rates are also used to monitor questing mosquitoes. Traps designed to collect gravid mosquitoes include traps baited with water and grass, oak, or bacteria, depending on the target species (Ritchie et al. 2014). Resting traps and boxes collect blood-fed mosquitoes that are seeking a place to stop and digest (Panella et al. 2011). All of these methods rely on knowledge of mosquito biology and behavior to be effective. Researchers and technicians can use this information to determine the best times and methods of controlling mosquito populations, therefore preventing the highest volume of bites. There are no medical treatments for many mosquito-borne diseases, so bite prevention is the only way to guarantee public health and safety.

Reverse Transcriptase Loop Mediated Isothermal Amplification for Screening Pathogens in Vectors

A variety of methods for screening vectors for pathogens currently exist (Martin et al. 2000; Lambert et al. 2005; Bishop-Lilly et al. 2010) including culturing, gene amplification via polymerase chain reaction (PCR), nucleic acid sequence-based amplification (NASBA), self-sustained sequence replication (3SR), and strand displacement amplification (SDA). PCR for DNA amplification and reverse transcriptase (RT)-PCR for RNA amplification are one of the most reliable and sensitive methods of molecular screening (Bustin et al. 2005). RT-PCR relies on expensive equipment (including thermalcyclers) and is difficult to perform in the field. Thermalcyclers are very costly (~$20,000.00) and not likely to be purchased by many small, minimally funded public health departments. Methods such as 3SR and SDA, which do not use thermocyclers are often lacking in accuracy or flexibility (Notomi et al. 2000).

A novel method of molecular detection of pathogens is loop mediated isothermal amplification (LAMP) (Notomi et al 2000), that combines the sensitivity of PCR with the cost-effectiveness of isothermal methods like 3SR and SDA. LAMP makes use of two inner primers and outer primers that recognize six different regions of target DNA, which increases accuracy because amplification only occurs when all six regions are correctly recognized (Mori and Notomi 2009). LAMP can also be used to synthesize DNA from RNA by reverse transcriptase in order to screen for RNA-based viruses (Notomi et al. 2000).

LAMP has been used successfully to detect pathogens and was first used for food-borne illnesses, including *Salmonella* (Hara-Kudo et al. 2005; Techathuvanan and D'Souza 2012) and

Escherichia coli (Hill et al. 2008). LAMP also has been used in the detection of HIV-1 (Curtis et al. 2008), severe acute respiratory syndrome (SARS), coronavirus (Hong et al. 2004), tuberculosis (Iwamoto et al. 2003), West Nile virus and dengue virus serotypes 1-4 (Li et al. 2011), and many others. LAMP is performed under isothermal conditions, and expensive thermocyclers are not necessary (Notomi et al. 2000), which means that tests can be performed by public health departments, or in field or temporary clinics (Mori and Notomi 2009). Isothermal assays have previously been used as a diagnostic tool in clinical settings in developing countries where resources and funding were limited (Boehme et al. 2007).

The LAMP reaction is similar to other molecular screening methods, such as PCR, by requiring the use of primers, an amplification buffer, DNA polymerase, and free nucleotides. In addition to the extra primers, the LAMP reaction requires a *Bst* polymerase that is more appropriate to isothermal conditions than the thermophilic *Taq* polymerase often used in PCR. The addition of betaine to the reaction may prevent base stacking and stabilize GC-rich sequences and improve strand displacement by *Bst* (Henke et al. 1997, Notomi et al. 2000, source). The addition of RTase is also necessary for use in screening RNA, t. Magnesium sulfate is added for production of magnesium pyrophosphate which is visualized by amount of turbidity (Mori et al. 2001) and is an alternative to visualization of a DNA band on a gel (standard RT-PCR) or amplification through the use of flourogenic probes (real-time RT-PCR).

Justification

Risk of severe complications or death from mosquito-borne disease is much lower in developed nations compared to many other parts of the world (Reiter et al. 2003). However, developed countries lack vaccinations and treatments for many of these serious pathogens and basic biological knowledge of the vectors is missing. . Mosquito control is a primary tool for disease prevention. Since avoidance of mosquitos is not always possible. Many mosquito species act as vectors for pathogens and not all are active at the same time. It is not practical for people to stay indoors all day and all night for the entire mosquito season every year. Worobey et al. 2013 even suggested that this avoidance is a contributing factor in childhood obesity. If children are not playing outside for fear of mosquito bites, they are not getting the exercise they need. Improving our understanding of mosquito behavior and activity is crucial to improving mosquito control methods which will lead to increased health and wellbeing of children particularly in southern Appalachia.

In 2013, Tennessee had the highest number of LACV cases in the country (USGS Disease maps 2013). In addition to the immeasurable human loss that can be associated with LACV, the economic impact on families is also significant. Haddow et al. (2011) suggested that those most at risk for contracting the virus are in lower-income communities, often with less than a high school diploma, who may be greatly impacted by high immediate and long-term medical costs associated with the disease. The direct medical costs, per person, caused by La Crosse encephalitis (LACE) may average over $30,000. With long-term neurological symptoms, the costs can exceed $40,000 per person (Utz et al. 2003). High economic impacts especially in low-

income areas and the potential for pediatric fatalities, require effective (efficient and accurate) diagnostic and prevention methods. Unfortunately, LACV may be misdiagnosed as enteroviral meningitis or herpes simplex encephalitis without accurate methods of vector surveillance and virus screening (McJunkin et al. 2001). The development of an RT-LAMP kit for screening for LACV is necessary for providing a single, multi-purpose method of screening mosquitoes and reservoir hosts for the virus in the field. Field surveillance is important because it allows rapid virus identification and permits quick information sharing to people at risk and neighboring communities. The low comparative cost of RT-LAMP that is rapid and accurate could reduce the cost of diagnostics and, in turn, reduce patient costs (Mori and Notomi 2009).

The goal of this project is, 1) to increase understanding of mosquito behavior in order to improve current mosquito monitoring and control methods and 2) to design and develop a successful RT-LAMP assay for effective and efficient screening of LACV in laboratory, field, and clinical settings. An improved understanding of diurnal and nocturnal vector activity will help health departments and mosquito control districts target certain vectors and, certain viruses more effectively. This information will also be shared with the public so individuals can make informed decisions about summertime activities and personal protection. An effective RT-LAMP design will be used to screen mosquitoes for LACV in specific such as around elementary schools. It will also provide public health workers, researchers, and control specialists with a useful diagnostic and monitoring tool that is inexpensive, accurate, and able to provide results in real time. Creating and distributing RT-LAMP assays for LACV will also allow a greater number and diversity of people to learn about these techniques and screen for the virus. The

methods can be applied to other virus screening in the future. Ultimately, this project will provide health departments and mosquito abatement districts with the necessary tools to decrease the spread of LACV and, as such, greatly improve public health and safety.

Objectives

The overall objective is to improve surveillance methods for LACV and vectors, ultimately leading to enhanced prevention and control of the disease.

Objective 1: Determine the temporal activity of LACV vectors, especially relating to typical working hours, in Knox County, TN. I will test the hypothesis that vector presence and questing or oviposition activity differs throughout the day. The null hypothesis is that there is no difference in activity of mosquitoes at any time; the alternate hypotheses are that there are differences either by species, trapping method, or both.

Objective 2: Develop a RT-LAMP diagnostic assay specific to LACV that will be effective in the field. I will test the hypothesis that an accurate and cost-effective virus screening method can be developed, and will be comparable (or better) compared to the gold standard, RT-qPCR. The null hypothesis is that the new method will not be better or less expensive; conversely, the alternate hypotheses are that it will be more effective than RT-qPCR or that it will be at least as effective, as well as less expensive and time consuming.

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2. IMPROVING SURVEILLANCE OF LACV VECTORS IN EASTERN TENNESSEE

ABSTRACT

In eastern Tennessee, La Crosse virus (LACV) is the most commonly diagnosed arbovirus and all symptomatic cases are in children under 16 years. A better understanding of vector behavior is necessary to prevent pathogen transmission by preventing bites and aiding mosquito control. This study identified questing and oviposition activity of LACV vectors, as they relate to traditional working hours. Nineteen sites were selected and sampled three times between June-September 2015. Six traps (two CDC questing traps baited dry ice, two CDC questing traps baited with dry ice and a chemical lure, and two CDC gravid traps baited with grass-infused water) operated at each site for a total of 48 hrs. Traps were refreshed and collection nets were changed each morning at 9:00 and evening at 17:00. Mosquitoes from the same site, same trap type, and same time-period were pooled to minimize zero counts and were then identified to species. The mean number of each LACV vector was compared by collection time (9:00 to 17:00 vs 17:00 to 9:00), collection trap (the three above traps), and collection trap x time interactive effects in a randomized block design multivariate analysis of variance. All LACV vectors (*Aedes triseriatus*, *Ae. albopictus*, and *Ae. japonicus*) were active throughout the 48hr sampling period, but significantly more were collected from 17:00-9:00 than from 9:00-17:00. Significantly more *Ae. japonicus* were collected in gravid traps indicating the need for improved *Ae. japonicus* questing traps. In this study we identified that the best method to collect questing LACV vectors is use of the CDC-trap baited with dry ice and lure operating from 17:00-9:00; this is also the time mosquitoes should be avoided. Information gathered by this study will improve vector surveillance and minimize pathogen transmission in east Tennessee.

Keywords: *Aedes,* La Crosse virus, mosquito activity, questing, oviposition

INTRODUCTION

Mosquitoes transmit a variety of pathogens that are difficult to accurately diagnose and expensive to treat (McJunkin et al. 2001, Reiter et al. 2003); consequently, understanding mosquito questing and oviposition activity is key to preventing mosquito-borne disease transmission (Bonds 2012). Knowing when biological behaviors occur, such as questing and ovipositing, helps prevent pathogen transmission (prevent bites) and reduces future populations (prevent offspring from developing).

Mosquito control districts, pest management professionals, and public health departments must take into account mosquito activity when implementing control efforts; consequently, they must share accurate and relatable information on mosquito activity to their clients (the public). Technicians, both private and public, must be able to understand when mosquitoes are biting and when they are ovipositing so they know when to apply control and monitoring measures. Technicians must also relay this information to their clients, so clients can implement bite prevention tactics by applying repellant or wearing long sleeves. This information will not be the same for every vector because different mosquito species have different oviposition and questing behaviors. Traps are often used to evaluate mosquito activity; gravid traps attract mosquitoes ready to oviposit and can target different species by varying water infusions , while questing traps target biting females and can target different species by varying placement and attraction odors (Williams and Gingrich 2007, Obenauer et al. 2010). For instance, questing traps intended to collect *Culex* mosquitoes should be placed higher into tree canopies since *Culex* prefer to feed

on birds and questing traps for *Aedes* mosquitoes should be placed at ground level because these mosquitoes tend to feed on mammals (Niebylski et al. 1994; Anderson et al. 2004).

Studies relating to the daily activity of different mosquitoes have been conducted, but not in southern Appalachia where *Aedes* mosquitoes are abundant. Both *Ae. triseriatus* and *Ae. albopictus* are considered diurnal biters (Robertson and Hu 1935; Clark et al. 1985), while *Ae. japonicus* is likely biting during crepuscular times (Land et al. 1999). Clark et al. (1985) observed diurnal landing rates of *Aedes triseriatus* during a summer in Illinois using human landing rates and found that mosquitoes were active throughout the daylight hours, without noting a time of obvious increase in activity. Chadee and Martinez (2000) measured *Aedes aegypti* activity in both urban and rural sites in Trinidad and determined that urban mosquitoes were more often diurnal, while rural mosquitoes were more often nocturnal which suggests that findings under certain conditions may not mirror findings under other conditions. For example, ULV sprays targeting West Nile virus (WNV) vectors are carefully timed to correspond with the nocturnal activity of *Culex* species since the treatment needs to make direct contact with flying mosquitoes (Bonds 2012). Another study determined that the use of nighttime ultra-low volume (ULV) sprays were successful in reducing populations of known diurnal *Ae. albopictus* in the northeastern United States (Farajollahi et al. 2012). In addition to the increased risk of pathogen transmission by these vectors, nuisance activity alone can directly affect the well-being of human hosts (Worobey et al. 2013). It is important to consider mosquito activity, when implementing control measures because this information determines when certain traps and control measures will be most effective (Reddy et al. 2007).

In the Appalachian region, La Crosse virus (LACV) is a medically important arbovirus and is transmitted via the bite of infected *Aedes* mosquitoes, including the primary vector *Ae. triseriatus*, and predicted secondary vectors *Ae. albopictus* and *Ae. japonicus* (Watts et al. 1973; Gerhardt et al. 2001; Bevins 2007; Hollidge and Soldan 2010). Children under age 16 are most at risk for severe disease, which can cause permanent neurological damage and, in some cases, death (McJunkin et al. 2001; Lambert et al. 2015). There are currently no vaccines or effective treatments for LACV (McJunkin et al. 1997), making accurate mosquito control and bite prevention methods essential to combating this disease. Avoiding and preventing bites by *Culex* mosquitoes to prevent WNV will be different from avoiding *Aedes* mosquitoes to prevent LACV (Clark et al. 1985; Gray et al. 2011).

In this study, we compared LACV vector activity during work hours (defined here as 9:00–17:00) and off work hours (defined here as 17:00-9:00) using trapping methods known to target questing and oviposition behaviors (Urquhart et al. 2016). Our goal was to provide insight in the development of future mosquito control and monitoring techniques by focusing on "typical" working hours; those that can be related to both technicians and to the public. We wanted techniques to be minimally disruptive to the current working schedules of mosquito control technicians, pest management professionals, public health departments, and local citizens. The objective of this study was to characterize the temporal activity of LACV vectors in order to improve mosquito surveillance, aid in management and control efforts, and to assist with bite prevention, which would aid in disease prevention. We tested the hypothesis that each LACV vector has a different temporal window for questing and oviposition activity and that this would be reflected in collection methods.

MATERIALS AND METHODS

Experimental Design

In eastern Tennessee, we chose 19 cemeteries as sites for monthly mosquito monitoring because cemeteries are known effective sites for collecting LACV positive mosquitoes (Trout Fryxell et al. 2015). Sites varied in size, vegetation, grave marker headstones (flat, bevel, slant, monuments, and ledgers), grave floral arrangements, and age. Small mammals, including squirrels (*Sciurus carolinensis*) and chipmunks (*Tamias striatus*), known reservoir hosts for LACV, were present at all of the sites. We did not alter any of the sites. Eighteen of the cemeteries sampled were located within a mile of schools of which sixteen were near elementary schools; picnic areas or play structures were present at four of the sites. This is particularly important because of the risk LACV poses to children. Additionally, nine of the sites were located within visual distance of auto and used tire shops where tires were observed outside, but tires were not inspected for immature mosquito development. All of the sites were located within a mile of varying bodies of water including rivers, lakes, creeks, and small ponds, and seven of the sites contained graves with built-in vases in which the investigators observed live mosquito larvae. We blocked the sites into four groups based on cardinal directions, and one block was visited each week until all four blocks were visited. We sampled each site three times over 12 weeks (22 June 2015 to 29 September 2015), each for 48 consecutive hours.

Environmental Factors

We recorded environmental conditions including temperature, relative humidity, and wind speed during each trap set-up and take down and determined means. We also obtained twilight hours, described as two hours before and after sunrise and sunset (Timeanddate.com). We recorded the mean temperature, relative humidity, wind speed, and time of sunrise and sunset using a Kestrel 3000® (KestrelMeters, Birmingham, MI) pocket wind meter.

Mosquito Monitoring

We chose three adult trapping methods based on results from Urquhart et al. (2016) and placed them in areas of dense vegetation. We used two questing traps, a CDC miniature light trap (Model number 512 John W. Hock Company, Gainesville, Florida) (CDC- CO_2) with the light removed and baited with ~900g of dry ice (carbon dioxide, CO_2) and another CDC trap baited with dry ice and an additional BioGents (BG) lure tied approximately 7-10 cm above the trap fan (CDC-CO_2lure) (Biogents through BioQuip Products, Rancho Dominguez, CA). The third trap we used was a CDC gravid trap (Model Number 1712 John W. Hock Company, Gainesville, FL) baited with ~2.2 L of an infusion made from grass soaked in warm water.

To maximize collections, we allowed traps to operate at each site for two days. To distinguish diurnal from nocturnal/crepuscular activity, we changed collection nets four times. Traps were set first at 9:00 and then collection nets were removed and baits refreshed at 17:00. We refreshed traps twice during work hours (9:00-17:00) and twice during after-work hours (17:00-9:00).

After retrieving collection nets, we stored mosquitoes within the nets in coolers lined with frozen ice packs to keep specimens alive prior to identification. Upon return to the laboratory, we aspirated mosquitoes from the nets, organized them into cups and provided a sugar solution in the form of Gatorade® (PepsiCo, Purchase, NY) until they could be processed (~12-24 hrs later). We exposed live mosquitoes to trimethylamine (Fisher Scientific), so we could paralyze them for identification to sex and species (Darsie and Ward 2005). We then sorted them by collection site, date, trap type, "day" or "night" collection, species, and sex.

Statistical Analysis

The data was analyzed using a mixed model analysis for a randomized block design (RBD) multivariate analysis of variance (MANOVA) with repeated measures. The response variables included the number of *Ae. triseriatus*, *Ae. albopictus*, *Ae. japonicus*, and the combined total counts. Site was the random block factor, trap was the between subject factor, and time was the within subject factor. The ANOVA test for each response variable was adjusted using a Bonferroni correction with a significance level of 0.0125. In all instances, data were rank transformed because ANOVA assumptions of normality and equal variance were violated. Post hoc multiple comparisons among levels of trap, time, and trap and time interaction were conducted with Tukey's adjustment and statistical significance was identified at the 0.05 level. All analysis was conducted with PROC GLIMMIX in SAS 9.4 TS1M3 (SAS Institute Inc., Cary, NC).

RESULTS

Environmental Factors

Temperatures ranged from 10.7 ºC during the week of 15 September to 34.8 ºC during the week of 22 June with a mean of 22.5 ºC (± 0.25 ºC). Relative humidity during the study averaged 74% (± 0.69 %RH) with a low of 44 %RH and a high of 99 %RH. Wind speed varied from 0 to 2.5 mph with a mean of 0.32 mph (± 0.02 mph). The average time of sunrise throughout the project was 6:50 and sunset was 20:22 indicating crepuscular activity ranged from 4:50 to 8:50 in the morning and from 18:22 to 22:22 in the evening. Since traps were changed twice during the day the 9:00-17:00 collections included 8 hrs of diurnal activity, and the 17:00-9:00 collections included 10 hrs of crepuscular activity and 6 hrs of nocturnal activity.

Mosquito Collections

We collected a total of 2,544 individual adult mosquitoes, comprised of 19 different species, consisting of *Ae. albopictus*, at 48.1% (1,223 specimens), *Ae. vexans* (Meigen) at 13.6% (346 specimens), *Cx. pipiens* complex at 9.8% (249 specimens), *Cx. erraticus* (Dyar and Knab) at 5.7% (146 specimens), *Anopheles punctipennis* (Say) at 5.3% (136 specimens), *Cx. restuans* (Theobald) at 4.5% (114 specimens), *Ae. triseriatus* at 3.5% (90 specimens), *Ae. atlanticus* (Dyar and Knab) at 2.9% (75 specimens), and *Ae. japonicus* at 1.9% (49 specimens) (Table 1). The remaining 11 species (4.6% representing 116 specimens) were *Ae. canadensis* (Theobald), *Ae. fulvus pallens* (Wiedemann), *Ae. trivittatus* (Coquillet), *An. quadrimaculatus* (Say), *An. walkeri* (Theobald), *Orthopodomyia signifera* (Coquillet), *Psorophora ciliata* (Fabricius), *Ps. ferox* (Von Humbolt), *Toxorhynchites rutilius septrilionis* (Coquillet), and *Uranotaenia sapphirini* (Osten

Table 1. Mosquito Collections. Mean number of mosquito species collected (± SE) over 12 weeks of collecting in Knox county, Tennessee. Mosquitoes were collected using three different trapping methods (CDC baited with dry ice, CDC baited with dry ice and an additional lure, and gravid traps baited with grass-infused water). Collections were made during work (9:00-17:00) or after-work (17:00-9:00) hours. Bolded species are important vectors in east Tennessee.

Mosquito Species	**CDC-trap (bait: dry ice)**		**CDC-trap (bait: dry ice & lure)**		**Gravid Trap (bait: grass-infused water)**		**Total**	
	9:00-17:00	17:00-9:00	9:00-17:00	17:00-9:00	9:00-17:00	17:00-9:00	9:00-17:00	17:00-9:00
	Mean number of mosquitoes collected per trap per trapping session (±SE)							
Aedes albopictus	**0.41 (± 0.043)**	**0.40 (± 0.049)**	**0.50 (± 0.115)**	**0.67 (± 0.096)**	**0.33 (± 0.048)**	**0.36 (± 0.057)**	**0.84 (± 0.107)**	**0.97 (± 0.096)**
Ae. atlanticus	0.01 (± 0.009)	0.14 (± 0.113)	0.11 (± 0.071)	0.16 (± 0.127)	0.00 (± 0.000)	0.16 (±0.158)	0.02 (± 0.012)	0.08 (±0.035)
Ae. canadensis	0.01 (± 0.009)	0.03 (± 0.035)	0.00 (± 0.000)	0.03 (± 0.021)	0.00 (± 0.000)	0.00 (± 0.000)	0.00 (± 0.000)	0.01 (± 0.006)

Table 1 Continued

Mosquito Species	CDC-trap (bait: dry ice)		CDC-trap (bait: dry ice & lure)		Gravid Trap (bait: grass-infused water)		Total	
	9:00-17:00	17:00-9:00	9:00-17:00	17:00-9:00	9:00-17:00	17:00-9:00	9:00-17:00	17:00-9:00
	Mean number of mosquitoes collected per trap per trapping session (±SE)							
Ae. f. pallens	0.00 (± 0.000)	0.01 (± 0.008)	0.00 (± 0.000)	0.00 (± 0.000)	0.00 (± 0.000)	0.00 (± 0.000)	0.00 (± 0.000)	0.003 (± 0.0019)
Ae. japonicus	**0.003 (± 0.0019)**	**0.002 (± 0.0022)**	**0.0 (± 0.00)**	**0.01 (± 0.007)**	**0.00 (± 0.000)**	**0.08 (± 0.019)**	**0.001 (± 0.002)**	**0.10 (± 0.03)**
Ae. triseriatus	**0.04 (± 0.009)**	**0.03 (± 0.095)**	**0.02 (± 0.010)**	**0.05 (± 0.014)**	**0.00 (± 0.000)**	**0.04 (± 0.011)**	**0.16 (± 0.021)**	**0.16 (± 0.021)**
Ae. trivittatus	0.02 (± 0.012)	0.04 (± 0.018)	0.00 (± 0.000)	0.01 (± 0.009)	0.00 (± 0.000)	0.01 (± 0.009)	0.003 (± 0.0021)	0.001 (± 0.0023)
Ae. vexans	0.07 (± 0.029)	0.78 (± 0.185)	0.25 (± 0.105)	1.39 (± 0.403)	0.10 (± 0.091)	0.03 (± 0.015)	0.08 (± 0.048)	0.07 (± 0.023)

Table 1 Continued

Mosquito Species	CDC-trap (bait: dry ice)		CDC-trap (bait: dry ice & lure)		Gravid Trap (bait: grass-infused water)		Total	
	9:00-17:00	17:00-9:00	9:00-17:00	17:00-9:00	9:00-17:00	17:00-9:00	9:00-17:00	17:00-9:00
	Mean number of mosquitoes collected per trap per trapping session (±SE)							
Aedes species	0.01 (± 0.009)	0.00 (± 0.000)	0.05 (± 0.055)	0.01 (± 0.008)	0.00 (± 0.000)	0.00 (± 0.000)	0.02 (± 0.02)	0.01 (± 0.010)
Anopheles punctipennis	0.02 (± 0.012)	0.44 (± 0.108)	0.02 (± 0.018)	0.53 (± 0.154)	0.01 (± 0.009)	0.01 (± 0.008)	0.01 (± 0.013)	0.01 (± 0.061)
An. quadrimaculatus	0.00 (± 0.000)	0.13 (± 0.071)	0.00 (± 0.000)	0.00 (± 0.000)	0.01 (± 0.009)	0.00 (± 0.000)	0.001 (± 0.0015)	0.04 (± 0.015)
An. walker	0.0 (± 0.00)	0.00 (± 0.000)	0.00 (± 0.000)	0.01 (± 0.009)	0.00 (± 0.000)	0.00 (± 0.000)	0.00 (± 0.000)	0.02 (± 0.011)
Culex. Erraticus	0.05 (± 0.024)	0.56 (± 0.127)	0.00 (± 0.000)	0.38 (± 0.133)	0.02 (± 0.018)	0.11 (± 0.048)	0.01 (± 0.005)	0.18 (± 0.056)

Table 1 Continued

Mosquito Species	CDC-trap (bait: dry ice)		CDC-trap (bait: dry ice & lure)		Gravid Trap (bait: grass-infused water)		Total	
	9:00-17:00	17:00-9:00	9:00-17:00	17:00-9:00	9:00-17:00	17:00-9:00	9:00-17:00	17:00-9:00
	Mean number of mosquitoes collected per trap per trapping session (±SE)							
***Cx. pipiens* complex**	**0.01 (± 0.027)**	**1.35 (± 0.35)**	**0.03 (± 0.016)**	**0.46 (± 0.141)**	**0.03 (± 0.020)**	**0.19 (± 0.087)**	**0.01 (± 0.005)**	**0.35 (± 0.059)**
Cx. restuans	0.02 (± 0.012)	0.53 (± 0.206)	0.00 (± 0.000)	0.36 (± 0.134)	0.00 (± 0.000)	0.03 (± 0.015)	0.02 (± 0.001)	0.003 (± 0.0021)
Culex species	0.0 (± 0.00)	0.00 (± 0.000)	0.00 (± 0.000)	0.11 (± 0.069)	0.00 (± 0.000)	0.03 (± 0.019)	0.00 (± 0.000)	0.04 (± 0.022)
Orthopodomyia signifera	0.0 (± 0.00)	0.00 (± 0.000)	0.00 (± 0.000)	0.01 (± 0.009)	0.00 (± 0.000)	0.00 (± 0.000)	0.00 (± 0.000)	0.003 (± 0.0021)
Psorophora ciliate	0.0 (± 0.00)	0.01 (± 0.008)	0.00 (± 0.000)	0.02 (± 0.012)	0.00 (± 0.000)	0.00 (± 0.000)	0.00 (± 0.000)	0.01 (± 0.008)

Table 1 Continued

Mosquito Species	CDC-trap (bait: dry ice)		CDC-trap (bait: dry ice & lure)		Gravid Trap (bait: grass-infused water)		Total	
	9:00-17:00	17:00-9:00	9:00-17:00	17:00-9:00	9:00-17:00	17:00-9:00	9:00-17:00	17:00-9:00
	Mean number of mosquitoes collected per trap per trapping session (±SE)							
Ps. ferox	0.08 (± 0.078)	0.06 (± 0.029)	0.02 (± 0.013)	0.02 (± 0.009)	0.00 (± 0.000)	0.01 (± 0.009)	0.03 (± 0.027)	0.05 (± 0.009)
Toxorhynchites rutilus septentrionalis	0.0 (± 0.00)	0.00 (± 0.000)	0.00 (± 0.000)	0.00 (± 0.000)	0.00 (± 0.000)	0.02 (± 0.012)	0.00 (± 0.000)	0.001 (± 0.0002)
Uranotaenia sapphirinia	0.01 (± 0.009)	0.00 (± 0.000)	0.01 (± 0.009)	0.00 (± 0.000)	0.00 (± 0.000)	0.00 (± 0.000)	0.003 (± 0.003)	0.003 (± 0.003)
Total	0.05 (± 0.008)	0.15 (± 0.013)	0.06 (± 0.013)	0.16 (± 0.016)	0.04 (± 0.006)	0.06 (± 0.009)	0.05 (± 0.005)	0.59 (± 0.0074)

Sacken). Damaged *Aedes* and *Culex* specimens comprised less than 1% of the collection and were not included in analyses. Mosquito collections peaked the week of July 6th (369 specimens) and were fewest during the week of June 28 (113 specimens) While no species were collected only from 900-1700hrs, five species were collected only from 1700-900hrs (*Ae. fulvus pallens*, *An. walkeri*, *O. signifera*, *Ps. ciliata*, and *Tx. r. septentrionalis*), but were few in number to make statistical comparisons.

LACV Vector Collections

We collected a total of 1,336 LACV vectors from all traps during all hours. The mean number of individual mosquitoes collected (±SE) for each time effect, trap effect, and trap by time interaction effect for each LACV vector species (*Ae. triseriatus*, *Ae. albopictus*, and *Ae. japonicus*) and the combined total for all vectors are presented in table 2. The results of all three vector species combined had a significant trap effect ($F = 9.32$; df = 2, 36; $P = 0.0005$), time effect ($F = 94.43$; df = 1, 1281; $P < 0.0001$), and trap x time effect ($F = 6.68$; df = 2, 1281; $P = 0.0013$). The CDC trap baited with dry ice and the BG-lure collected the most LACV vectors from 17:00-9:00 than the other trap x time combinations ($F = 6.68$; df = 2, 1281; $P = 0.0013$).

Significantly more *Ae. triseriatus* were collected between 17:00-9:00 (0.44 ± 0.01 mosquitoes) than 9:00-17:00 (0.019 ± 0.01) ($F = 16.73$; df = 1, 73; $P < 0.0001$). There was no significant trap ($F = 0.83$; df = 2, 36; $P = 0.44$) or trap by time interaction effect ($F = 2.01$; df = 2, 1281; $P = 0.134$).

Table 2. Statistical Analysis. Mean number of individual mosquitoes collected (±SE) followed by statistics for each time effect, trap effect, and trap by time interaction effect for each LACV vector species (*Ae. triseriatus*, *Ae. albopictus*, and *Ae. japonicus*) and the combined total for all vectors. Three traps (CDC baited with dry ice, CDC baited with dry ice and an additional lure, and gravid traps baited with grass-infused water) were set at 19 different cemeteries over 12 weeks in Knox county, Tennessee. Collections were made during work (9:00-17:00) or off-work (17:00-9:00) hours.

		Aedes triseriatus	***Aedes albopictus***	***Aedes japonicus***	**Overall**
		Time Effect			
9:00 - 17:00		0.03 ± 0.009 A	0.40 ± 0.049 A	0.002 ± 0.0022 A	0.15 ± 0.018 A
17:00 - 9:00		0.04 ± 0.011 B	0.36 ± 0.057 B	0.08 ± 0.019 B	0.16 ± 0.021 B
	Statistic	$F = 16.73$; df = 1, 73; $P < 0.0001$	$F = 9.02$; df = 1, 1281; $P = 0.0027$	$F = 22.17$; df = 1, 1281; $P < 0.0001$	$F = 94.43$; df = 1, 1281; $P < 0.0001$
		Trap Effect			
CDC-trap (bait: dry ice)		0.04 ± 0.009 A	0.41 ± 0.043 A	0.003 ±0.0019 A	0.15 ± 0.015 B
CDC-trap (bait: dry ice & lure)		0.035 ± 0.009 A	0.59 ± 0.075 A	0.007 ± 0.0035 A	0.21 ± 0.026 A

Table 2. Continued

		Aedes triseriatus	*Aedes albopictus*	*Aedes japonicus*	**Overall**
Gravid Trap (bait: grass-infused water)		0.02 ± 0.005 A	0.35 ± 0.04 A	0.04 ± 0.009 B	0.14 ± 0.013 C
	Statistic	$F = 0.83$; df = 2, 36; $P = 0.44$	$F = 1.65$; df = 2,36 ; $P = 0.2072$	$F = 8.77$; df = 2, 1281; $P = 0.0008$	$F = 9.32$; df = 2, 36; $P = 0.0005$
		Trap x Time Effect			
CDC-trap (bait: dry ice)	9:00 - 17:00	0.04 ± 0.014 A	0.42 ± 0.071 A	0.005 ± 0.0032 A	0.11 ± 0.016 A
	17:00 - 9:00	0.03 ± 0.009 A	0.4 ± 0.05 A	0.002 ± 0.0022 A	0.15 ± 0.018 B
CDC-trap (bait: dry ice & lure)	9:00 - 17:00	0.02 ± 0.010 A	0.5 ± 0.12 A	0.00 ± 0.000 A	0.17 ± 0.039 A
	17:00 - 9:00	0.05 ± 0.014 A	0.67 ± 0.096 A	0.01 ± 0.007 A	0.24 ± 0.033 B
Gravid Trap (bait: grass-infused water)	9:00 - 17:00	0.00 ± 0.000 A	0.33 ± 0.048 A	0.00 ± 0.000 A	0.11 ± 0.016 A
	17:00 - 9:00	0.042 ± 0.0108 A	0.36 ± 0.057 A	0.04 ± 0.011 B	0.16 ± 0.206 C
	Statistic	$F = 2.01$; df = 2, 1281; $P = 0.134$	$F = 2.57$; df = 2, 1281; $P = 0.0770$	$F = 15.38$; df = 2, 1281; $P < 0.0001$	$F = 6.68$; df = 2, 1,281 ; $P = 0.0013$

The mean number of *Ae. albopictus* mosquitoes collected from 9:00-17:00 (0.42 ± 0.05 mosquitoes) was significantly lower than those collected from 17:00-9:00 (0.48 ± 0.04 mosquitoes) ($F = 9.02$; df = 1, 1281; $P = 0.0027$). There was no significant trap effect ($F = 1.65$; df = 2, 36; $P = 0.2072$) or trap by time interaction effect ($F = 2.57$; df = 2, 1281; $P = 0.0770$).

There was a significant trap, time, and trap by time interaction effect in the case of *Ae. japonicus*. More *Ae. japonicus* were collected from 17:00-9:00 (0.032 ± 0.007 mosquitoes) than from 9:00-17:00 (0.002 ± 0.001) ($F = 22.17$; df = 1, 1281; $P < 0.0001$). More *Ae. japonicus* were also collected from the gravid trap than any other trap ($F = 8.77$; df = 2, 1281; $P = 0.0008$). There was also a significant trap by time effect with *Ae. japonicus*, such that the gravid trap collected significantly more *Ae. japonicus* (0.49 ± 0.09 mosquitoes) from 17:00-9:00 (0.08 ± 0.019 mosquitoes) than all of the other trap by time combination effect ($F = 15.38$; df = 2, 1281; $P < 0.0001$).

DISCUSSION

In this study we observed that LACV vectors were primarily active from 17:00 to 9:00, aligning with after-work hours indicating caution should be taken during those times to avoid bites and prevent oviposition. The most effective trapping methods and times did not vary by species indicating after work collections are optimal and CDC questing traps with CO_2 and the BG lure will capture all vectors. We can use this information to educate citizens about how to prevent mosquito bites and prevent LACV transmission. Ideally monitoring programs for LACV vectors should use both questing and gravid traps running for 24 hours since *Ae. japonicus* was primarily collected with this trap. Programs focusing on individual vectors may differ, depending on the

target species. Knowing the best locations, traps, and times to collect vectors is crucial for effective LACV monitoring (Trout Fryxell et al. 2015, Urquhart et al. 2016).

The primary vector, *Ae. triseriatus*, is both host-seeking and ovipositing more often during after-work hours (17:00-9:00) than work hours (9:00-17:00) because all three traps collected *Ae. triseriatus* mosquitoes more often from 17:00-9:00 than from 9:00-17:00. This suggests possible crepuscular and/or nocturnal activity. These findings differ from previous studies depicting *Ae. triseriatus* as a diurnal species that is continually host-seeking (results based on human landing rates) for a period between 7:40 and 20:40 (Clark et al.1985). This could be due to a difference in temperature during midday between the Appalachian region and Illinois, where Clark et al. (1985) completed their study. Also, the use of a human as a trap provides multiple layer of attraction missed by our manufactured traps.

The most abundant vector, *Ae. albopictus,* was also found to be ovipositing and host-seeking most often during after-work hours (17:00-9:00). Significantly more *Ae. albopictus* were collected using all trapping methods during that time. While previous studies have determined or declared *Ae. albopictus* to be a diurnal species (Farajollaha 2012), Yee and Foster (1992) observed host-seeking activity by *Ae. albopictus* during both daytime and nighttime hours. Here, our data may corroborate Yee and Foster (1992) that *Ae. albopictus* is active during both daylight and nighttime hours, since the after-work traps were set before sunset and collected before sunrise. Fewer *Ae. albopictus* collected during work hours could be due to the shorter time period, or the weather patterns (heat) of eastern Tennessee.

Aedes japonicus was the only LACV vector with specific trap and time effects; it was collected primarily during after-work hours (17:00-9:00) with the gravid trap; assumingly ovipositing. Here questing activity cannot be properly assessed for *Ae. japonicus* because the questing traps collected this species in so few numbers (total = 49 specimens). Currently, little is known regarding temporal activity of *Ae. japonicus*. Although, observations of the physiology of the *Ae. japonicus* eye by Land et al. (1999) suggests crepuscular activity is common in this species, but this has not been corroborated by trapping studies or daytime vs. nighttime collections. In the future, further studies on the behavior of this species, including habitat preference and host-seeking behavior, must be done to aide in the design and implementation of better monitoring programs.

Vector monitoring and control programs must consider the typical work day because in the United States, the average citizen works during the hours of 9:00 to 17:00. Understanding the activity of mosquitoes as it relates to the workday will help promote educational programs on prevention and control of mosquitoes. Because some daylight hours are included within the17:00-9:00 trapping times, this study does not necessarily provide information on exact temporal activity; however, activity of LACV vectors measured during typical working hours will determine when their behavior could be most effectively exploited by monitoring programs. The Knox county health department in east Tennessee often sets traps during the day (9:00-17:00) and applies treatments after-work (17:00-9:00), knowing mosquitoes are primarily active after 15:00 indicates monitoring strategies can begin around 13:00 and population control strategies can begin around 17:00; such that traps should be set at the end of the work day to collect nocturnal and crepuscular species and picking them up in the morning or setting them

first thing in the morning and collecting them at the end of the day. This information will also help aid private citizens on when and how to effectively prevent mosquito bites and control mosquitoes around their homes.

Overall well-being of children and families in the Appalachian region depends on determining and implementing best practices for monitoring and control of LACV. Children younger than 16 are at the greatest risk for severe LACE (Tolle 2009), and these children may develop permanent neurological problems which places an additional financial and immeasurable emotional burden on affected communities (McJunkin et al. 2001). Effective vaccines against LACV do not exist, and LACE diagnosis can be difficult as clinical symptoms mirror those of other viruses; therefore, mosquito control is the best method for combatting this disease (McJunkin et al. 2001). The information collected in this study allows for more effective monitoring and control of LACV vectors, which will aide in the development of effective mosquito control, disease prevention, and protection of the health of children in eastern Tennessee.

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3. DEVELOPMENT OF A NOVEL MOLECULAR METHOD FOR LA CROSSE VIRUS DETECTION

ABSTRACT

La Crosse virus (LACV) is the leading cause of arboviral encephalitis in children and the most severe cases often occur in individuals under 16-years-of age; severe cases are associated with permanent brain damage or rarely death. LACV is transmitted via the bite of the primary vector, *Aedes triseriatus*, and secondary vectors *Ae. albopictus* and *Ae. japonicus.* No vaccines or antivirals exist, making accurate surveillance and prevention methods crucial for protecting human health. The standard molecular method for LACV screening is reverse transcriptase polymerase chain reaction (RT-qPCR), an effective but time consuming and expensive procedure that is difficult for mosquito abatement districts and county health departments to preform because they often lack equipment, expertise, and funding for these tests. We hypothesized that an economical, isothermal, sensitive, specific, and faster molecular method for the detection of LACV from vectors and reservoirs could be developed. We tested CDC-Ft. Collins positive controls using the standard RT-qPCR protocol and compared it with a newly designed procedure with varying concentrations of reagents, including primers, buffer, and enzymes. We tested the novel reaction mix against RT-qPCR for efficacy by running a 10-fold dilution series to determine the lowest concentration of virus detectable by each method. Both the RT-qPCR and RT-LAMP methods reliably detected LACV at a 10^{-6} dilution (1.82^{-6} ng/µl) ($P > 0.05$, chi-square test). The RT-LAMP method is less expensive (~$1,000 for the RT-LAMP vs. $20,000 for the RT-qPCR) and takes only half the time (90 min for RT-LAMP compared to 180 min for RT-qPCR) which indicates we developed a novel LACV detection method that is cost-effective and rapid. Mosquito abatement districts and county health departments can use our diagnostic tool.

Keywords: RT-LAMP, La Crosse virus, *Aedes*, detection, Appalachia

INTRODUCTION

La Crosse virus (LACV) is a single stranded, spherical, enveloped, negative sense RNA virus, approximately 80-100nm long, containing small (S), medium (M), and large (L) segments of circular RNA (Gentsch and Bishop 1978). It is in the family *Bunyaviridae* (Elliott 1990) and is the causal agent of La Crosse encephalitis (LACE), which is the most commonly diagnosed mosquito-borne virus in children in North America. The virus was first isolated from the brain of a four-year-old girl in La Crosse, Wisconsin, in the 1960's (Thompson et al. 1965). The severe form of the disease can cause permanent neurological damage or death and can be difficult to diagnose because of its symptomatic similarity to both meningitis and herpes simplex encephalitis (McJunkin et al. 2001). Currently, no cure or effective vaccine exists, so vector control and virus surveillance are the best available preventative measures. LACV is transmitted by *Aedes* mosquitoes (Thompson et al. 1972). The primary vector is *Ae. triseriatus* and there are likely two secondary vectors (*Ae. albopictus* and *Ae. japonicus*) (Thompson et al. 1972; Watts et al. 1972; Gerhardt et al. 2001; Bevins 2007; Soldan et al. 2010; Harris et al. 2015). The most common amplifying hosts include eastern gray squirrels (*Sciurus carolinensis*) and eastern chipmunks (*Tamias striatus*), which are important for maintaining the virus in the environment (Pantuwatana et al. 1972; Ksiazek and Yuill 1977). LACV can also be transmitted both venereally and transovarially between *Ae. triseriatus* individuals (Miller et al. 1977; Thompson and Beaty 1978).

Low cost and effective methods are available for monitoring *Aedes* and *Culex* mosquitoes in a given area (Urquhart et al. 2016), but current viral screening methods are costly and time

consuming. La Crosse virus exposure can be detected from children using an Immunoglobin M (IgM) antibody capture enzyme linked immunosorbent assay (MAC ELISA), although the lack of an effective treatment makes prevention a greater priority (Calisher et al. 1986; Martin et al. 2000). A detection method that can be used both to determine the presence of LACV in the vector population before the virus spreads to humans as well as more effectively screen sick humans would aid in both prevention and diagnostics. Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) is the current gold standard for detecting LACV from field-collected *Aedes* mosquitoes (Lambert et al. 2005). While this method is sensitive and accurate, it requires the use of expensive thermalcyclers and reagents that many county level health departments and mosquito abatement districts cannot financially afford because of costs and labor (Mackay et al. 2002, Parida et al. 2004, Lambert et al. 2005, Heid et al. 2014). RT-PCR also takes several hours to complete and samples often have to be shipped to better-equipped laboratories. Thus, there is a critical need to develop an inexpensive, thermostable, and timely method for LACV surveillance that is at least as effective as RT-qPCR. The main objective of this project was to develop such a method for LACV detection.

Inexpensive and timely molecular detection methods for other pathogens, have been developed using a template method called loop mediated isothermal amplification (LAMP) or reverse transcriptase loop mediated amplification (RT-LAMP) in the case of RNA viruses (Notomi et al. 2000; Mori and Notomi 2009). This test has been used as rapid and cost-effective screening method for a variety of pathogens, including *Salmonella* (Hara-Kudo et al. 2005; Techathuvanan and D'Souza 2012)*, Escherichia coli* (Hill et al. 2008), HIV-1 (Curtis et al. 2008), Tuberculosis (Iwomato 2003; Boehme et al. 2007) and severe acute respiratory syndrome coronavirus (Hong

et al. 2004). Additionally, RT-LAMP methods have been developed for other arboviruses, including West Nile Virus, for which a commercial kit has been created, and Dengue fever, which is used diagnostically in developing countries (Parida et al. 2004, Parida et al. 2005). RT-LAMP is considered isothermal because the entire reaction runs at a single temperature, and requires only an inexpensive water bath or hot plate for pathogen detection (Teoh et al. 2013). Results are often obtained in 30 to 90 minutes and can be observed directly in the reaction tube through turbidity created by the production of magnesium pyrophosphate or through fluorescence (Parida et al. 2008). Our hypothesis was that LACV detection could be made simpler and more affordable by using RT-LAMP. In this study we developed an RT-LAMP assay for detecting LACV and compared the assay to the standard RT-PCR assay to determine end-point detection (Lambert et al. 2005).

MATERIALS AND METHODS

Virus Strain

A LACV original strain (MN 1960) positive control was obtained from the Centers for Disease Control vector biology laboratory. The laboratory prepared the sample in suckling mice and shipped it on dry ice to the University of Tennessee Medical and Veterinary Entomology Laboratory in Knoxville Tennessee. Once the sample arrived, the virus was stored at -80^{o}C until it was subjected to RNA extraction using the Qiagen Mini Viral RNA kit (Qiagen, Inc. Valencia CA, Catalog #52904). RNA extraction protocols from mosquitoes are in Appendix 1.

RT-LAMP assay design

The S-Segment of LACV genome was chosen for amplification via RT-LAMP because it is the most highly conserved region (Gentsch and Bishop 1978). LACV primers reported by Urquidi and Bishop (1992) were used as the two outer (F3 and B3) RT-LAMP primers. The basic local alignment search tool (BLAST) provided by the National Center for Biotechnology Information (NCBI) was used to design the forward inner primer (FIP), backward inner primer (BIP), forward loop primer (FLoop), and backward loop primer (BLoop).

The RT-LAMP reaction was optimized in a 25µl reaction containing 15U/µl avian myeloblastosis (AMV) (Invitrogen, Life Technologies, Grand Island, NY, catalog #12328019) tested at various volumes between 0.25 to 2µl, 8U *Bacillus stearothermopolis* DNA polymerase (*Bst*) (New England BioLabs Inc., Ipswich, MA, Catalog #M0275L) tested between 0.5 and 1.5µl, 5M betaine (Sigma Aldrich, St. Louis, MO, Catalog #B0300) between 0.5 and 5µl, 10mM dNTPs (Invitrogen, Life Technologies, Grand Island, NY, Catalog #18427013) between 5.25 and 10µl, 10X Thermopol Buffer (New England BioLabs Inc., Ipswich, MA, Catalog #M0275L) between 2 and 5µl, 100mM magnesium sulfate ($MgSO_4$) (New England BioLabs Inc., Ipswich, MA, Catalog #M0275L) tested between 3 and 6µl, and molecular grade water tested between 2.5 and 8.75µl. Additionally, 100µM of the above six primers were tested at volumes between 0.5 and 1µl (FIP/BIP), 0.05 and 0.125µl (F3/B3), and the two Loop primers were only tested at volumes of 0.25µl. The original LAMP design was tested using an Applied Biosystems Veriti Thermalcycler (ThermoFisher, Life Technologies, Grand Island, NY, Catalogue #4375786) to ensure consistency of temperature.

The reaction mixture was first tested 15 different times using varying volumes of reagents, primers, temperatures, and extension times. The reaction was tested at four different temperatures: 55ºC, 58ºC, 60ºC, and 63ºC. Protocols were also tested at 60 min, 75 min, and 90 min. Turbid samples were identified as positive and then 10 µl of that sample was confirmed for positivity via gel electrophoresis. RT-LAMP positive samples were verified by running a 2% agarose gel in a 50X TAE buffer (ThermoFisher Scientific, Life Technologies, Grand Island, NY, Catalog # B49) at 100 volts for 60-90 min, positives were confirmed if the band was 80-90 bp.

RT-LAMP use in water bath

The most regularly successful RT-LAMP assay was tested twice using the previously described reaction incubated in a 58ºC water bath for 90 min. The temperature fluctuated in the water bath between 59ºC and 61ºC. The initial denaturing was conducted in the thermalcycler.

Dilution series

To compare the RT-LAMP to the gold standard RT-qPCR, a dilution series on the RNA positive control was performed. For RT-qPCR, the M segment of the LACV genome was amplified on the BioRad Iq5 Real Time PCR Machine (BioRad, Hercules, CA, Catalog # 170-9753 and 170-8701) using the Qiagen Quantitect Probe RT-PCR kit (Qiagen, Venlo, Netherlands) predefined M-segment primers (Lambert et al. 2005), and Taqman probes by Thermo Fisher Scientific (Life Technologies, Grand Island, NY, Catalog #4316034) in a 25µl total reaction mix. A detailed method for RT-qPCR assay is in Appendix 2.

RNA from the positive control was quantified using a Nanodrop 1000 Spectrophotometer (ThermoFisher Scientific, Life Technologies, Grand Island, NY) and was identified at 1.82 ng/µl. Using the starting concentration and ending at 10^{-11} (1.82^{-11} ng/µl), a10-fold dilution series was compared in triplicate using RT-qPCR and RT-LAMP protocols to determine the lowest point of detection by both protocols. The reaction tubes were only available in strips of eight, so dilutions had to be run in groups of six, plus a negative and a positive control. We ran three replicates each through RT-qPCR and RT-LAMP that included a positive control followed by dilutions 10^{-1} through 10^{-6} and a negative water control. We also ran three replicates through each method that included the same positive and negative controls, as well as dilutions from 10^{-6} through 10^{-11}. As a result, we ran a total of three replicates for 10^{-1} through 10^{-5} and 10^{-7} through 10^{-11}, and six replicates of the 10^{-6} dilution. To compare the lowest point of detection in each assay, the number of times the 10^{-6} dilution was detected via RT-LAMP was compared to the number of times it was detected via RT-qPCR with a chi-square ($\alpha = 0.05$).

RESULTS

RT-LAMP assay design

Of the fifteen protocols evaluated, four produced turbid samples and the correct gel band (26.7% success). Of the successful four protocols, one protocol consistently produced turbid samples and gel bands on more than ten attempts and successfully produced positive samples at concentrations below the control concentration (1.82 ng/µl). One assay was successful at 60ºC, but the remaining three assays were only successful at 58ºC. Specifically, three failed assays were tested at 63ºC for 60 min, two were tested at 60ºC for 60 min, seven were tested at 60ºC for

90 min, two were tested at 55ºC for 90 min, two at 63ºC for 90 min, and one at 58ºC for 75 min. One successful assay worked at 60ºC for 90 min, but the same design failed a second attempt. All other successful assays worked at 58ºC for 90 min, but differed in volumes of reagents and primers.

The most successful assay used the following: 1.01µl of 15U/µl AMV, 1.32µl of 8U *Bst*, 3µl of 5M betaine, 6.25µl 20mM dNTPs, 2.5µl 10X Thermopol Buffer, 0.5µl 100µM FIP and BIP, 0.05µl 100µM F3 and B3, 0.25µl 100µM FLoop and BLoop, 3.32µl $MgSO_4$, and 1µl H_2O. These reagents were added to 5µl of viral RNA. The reagents were mixed on ice and RNA was heated for 1 min at 95ºC just prior to being added. To amplify the virus, the reaction mix (RT-LAMP mix and virus) was heated for 90 min at 58ºC. A detailed method for the successful RT-LAMP design is in Appendix 3.

RT-LAMP use in water bath

The protocol in the water bath successfully produced turbidity and the gel band. While the initial denaturation of the sample was completed in the thermalcycler at 95ºC for 1 min, before being moved to the water bath, this step could be replaced with a hot plate. Use of a water bath will significantly reduce the cost of screening.

Dilution series

Using the standard assay for RT-qPCR the mean cycle threshold (Ct) value (number of cycles required for the fluorescence signal to be detectable beyond background levels) for the positive control was 22 after running three replicates. Following this, concentrations had mean Ct values

of 24.3 at 10^{-1} dilution, 27 at 10^{-2}, 31.3 at 10^{-3}, 34 at 10^{-4}, 38 at 10^{-5}, and 39 at 10^{-6}, respectively. Positive sample was not detected via RT-qPCR beyond a dilution of 10^{-6}. When the RT-qPCR product was run through gel electrophoresis, bands were produced for 10^{-1} through 10^{-6} concentrations at five replicates. Bands were produced at 10^{-7} and 10^{-11} for one replicate each.

The RT-LAMP showed turbidity at all concentrations up to 10^{-6} for all three replicates, at 10^{-6} for five out of six replicates, and at concentrations as low as 10^{-10} for one out of three replicates. When the RT-LAMP product was run through gel electrophoresis, amplification was observed through 10^{-6} for two replicates and at 10^{-10} for one replicate.

The 10^{-6} dilution was the lowest concentration detected more than once by both methods. These results were compared using a chi-square test, which found both methods to be equally effective at detecting LACV at a 10^{-6} dilution ($P > 0.05$, chi-square test) (Table 3). This demonstrates that both the RT-qPCR and RT-LAMP are capable of amplifying LACV to concentrations of 10^{-6} (Fig 1).

Table 3. Molecular Methods Comparison. Chi-square contingency table comparing the number of successful and unsuccessful detections of La Crosse virus at a dilution of 10^{-6} by both RT-qPCR and RT-LAMP indicated no significant difference in detection. Observed values are recorded followed by expected values in parentheses.

	Gel Confirmation	Gel Contradiction	Total
RT-qPCR	5 (2.5)	1 (0.5)	6
RT-LAMP	5 (2.5)	1 (0.5)	6
Total	10	2	24

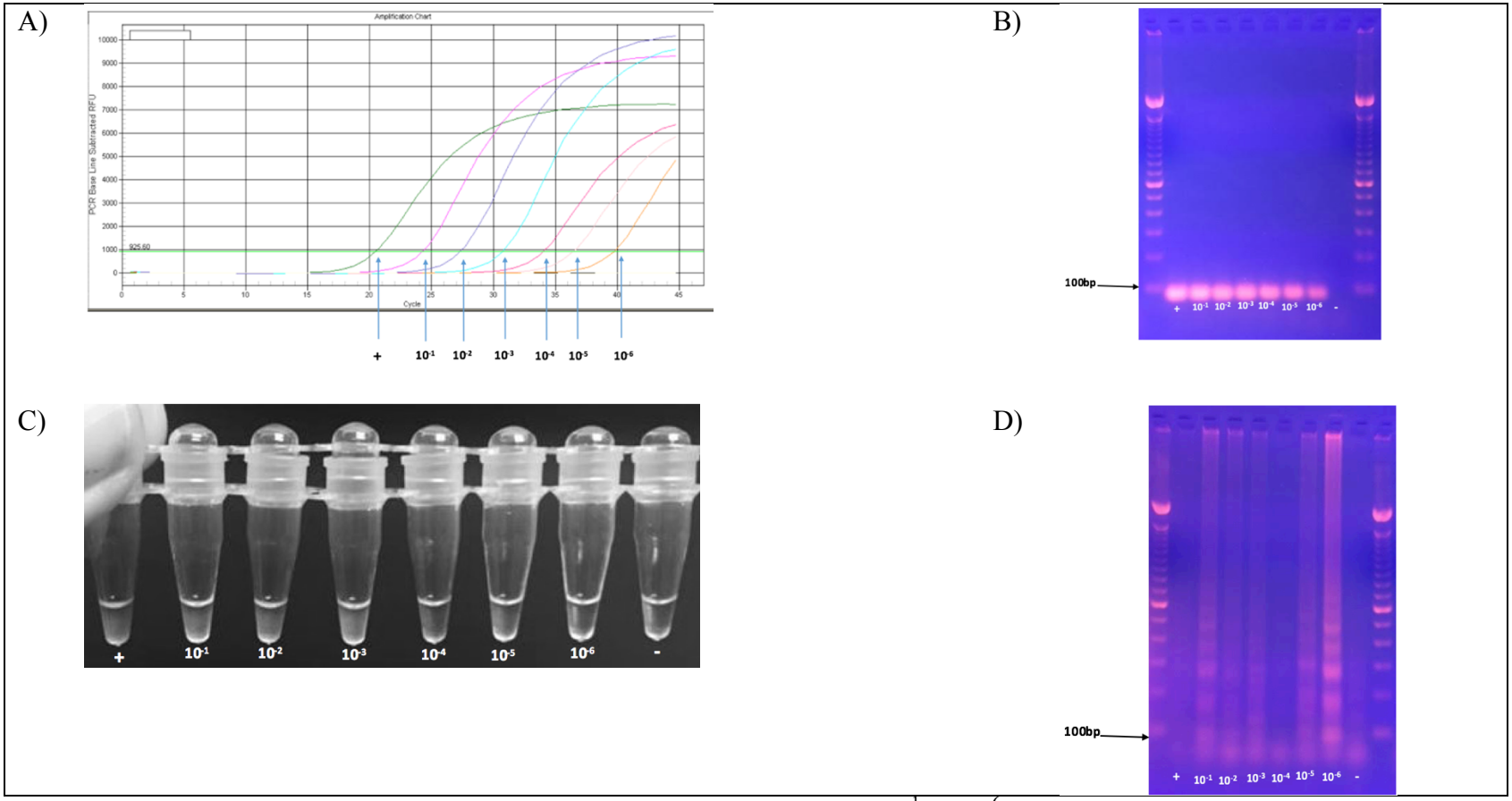


Fig 1. Dilution Results. Results of a 10-fold dilution series (concentrations 10^{-1} to 10^{-6} ng/µl) using RT-qPCR depicting positive control at a Ct value of 21 (A), gel confirmation of the RT-qPCR with bands at 83bp (100bp ladder template) (B), the newly developed RT-LAMP showing turbidity at all concentrations (C), and the RT-LAMP gel depicting bands of varying weights in a ladder pattern, with the bands at 83bp indicating the sample is positive for LACV (D).

DISCUSSION

In this study, we developed an RT-LAMP method for the detection of LACV that was comparable to the gold standard RT-qPCR; both successfully identified virus at 10^{-6} concentration (1.82^{-6} ng/µl). This novel method is less expensive than RT-qPCR in both equipment and reagents and it takes half the time to complete a run. The cost of the reagents to run 50 reactions with the RT-LAMP is approximately $500 while the cost of RT-qPCR reagents cost approximately $900. The equipment necessary to run these reactions varies greatly with a real-time PCR system costing between $15,000 (used) and $20,000 (new). A simple water bath, costs around $1,000 at the high end of pricing. This novel method has great potential for future applications and is accessible in variety of situations, including county health department laboratories, extension laboratories, and mosquito abatement districts. The reduced cost and reaction time also provides greater research opportunities. The eventual development of a commercial kit based on the RT-LAMP design may provide additional convenience and ease of use.

The lowest concentration of LACV that was detected via both RT-LAMP and RT-qPCR was 1.82^{-6} ng/µl. Both methods are equally reliable; however, additional replicates within the dilution series may indicate a further difference because at least one replicate at 1.82^{-6} ng/µl did not show up as positive with both procedures. With more replicates, it is possible that further differences will appear. While the RT-qPCR did not detect virus at any point beyond a 10^{-6} ng/µl dilution, the RT-LAMP detected virus in one replicate at 10^{-8}ng/µl, 10^{-9}ng/µl, and 10^{-10}ng/µl. The higher resolution of RT-LAMP may be the result of contamination so we recommend more replicates to

verify the detection of virus at lower concentrations. In the future, it would be beneficial to test at least ten replicates. Sensitivity and specificity must both be determined for both the RT-LAMP and RT-qPCR methods. Lambert et al. (2005) ran a dilution series on titrated LACV to compare the nucleic acid sequence based amplification (NASBA) method with RT-qPCR and also compared 17 mosquito pooled samples and 10 human samples. Similar methods should be used to compare the RT-LAMP with the RT-qPCR.

The RT-LAMP must also be tested against related mosquito-borne viruses to ensure no cross-reaction. This has been done to determine the accuracy of previously designed RT-LAMP methods, such as those for Japanese encephalitis virus (JEV), Dengue virus (DENV 1-4), and West Nile virus (WNV) (Toriniwa and Komiya 2006, Parida et al. 2004, Parida et al. 2005). This is necessary to be certain the primers designed for the method are specific to the virus of interest. Designing four to six primers for use in the reaction that are specific only to a single virus can be difficult.

Without an effective vaccine or antivirals available to prevent or treat LACE, mosquito control is crucial to LACE disease prevention. While monitoring methods used to detect the presence of LACV vector species collected in southern Appalachia have been identified (Urquhart et al. 2016), it is necessary to screen these vectors for the virus to most accurately assess risk to the community. Once evaluated, this assay can be used to prevent the development and spread of LACV-infected mosquitoes especially in areas with limited funding. While targeting vectors generally is helpful, disease prevention depends on the control of the individual mosquitoes harboring the virus. In Tennessee, many county level health departments do not have qPCR

machines and have to send samples to the state laboratory for screening or forego detection completely. By increasing accessibility to reliable methods for quickly detecting LACV in vectors, we can intercept the pathogen before it has a chance to put children at risk.

The RT-LAMP method developed in this study offers an opportunity for county level health departments to screen field-collected mosquitoes for LACV efficiently. In the future, this method can be adapted for screening reservoir hosts and possibly as a diagnostic test for patients. The eventual development of a commercial kit based on this protocol would provide additional opportunities for screening in potentially high-risk areas (ex. around elementary schools and daycare centers) with minimal technical training required. The ability to quickly and inexpensively screen for this virus in the field greatly increases opportunities for risk assessment in endemic areas and is very important for disease prevention.

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4. CONCLUSIONS

This thesis aids in the improvement of LACV vector surveillance and virus screening methods and, as a result, provides important opportunities for disease prevention. Increased understanding of LACV vector behavior improves monitoring programs and mosquito control. The development of a more efficient method of screening these vectors provides the chance for a more affordable and efficient assessment of virus prevalence. Improvements to vector and virus surveillance can be used by extension agents, health departments, and researchers. County level health departments and mosquito abatement districts will be able to increase screening and surveillance of LACV and provide information to the public faster so citizens can protect themselves against bites and better control mosquitoes around their homes. Academic researchers will be able to save money and time on screening samples, which will increase LACV research potential. Determining how and when to best target vectors (questing and ovipositing) and be able to quickly and inexpensively screen these vectors for LACV, increases overall knowledge of the pathogen and improves the health of children in Eastern Tennessee.

Vector Surveillance

The first objective of this study was to improve understanding of the activity of *Aedes* vectors in eastern Tennessee in relation to working hours in order to increase opportunities for vector control and disease prevention. Trapping methods were compared during work and off-work hours (900-1700 or 1700-900, respectively) all three vectors (*Ae. albopictus, Ae. japonicus* and *Ae. triseriatus)* were collected during all trap x time comparisons. *Ae. albopictus* was collected at all times and with all traps, but both *Ae. japonicus* and *Ae. triseriatus* were collected more often during off-work hours. This does not necessarily suggest strictly nocturnal activity because off-

work traps ran during some daylight hours. According to questing trap collections, questing activity was exhibited equally by all three vectors during both work and off-work hours. Oviposition activity, determined by gravid trap collections, was exhibited by *Ae. albopictus* at all hours, but only by *Ae. japonicus* and *Ae. triseriatus* were collected primarily during off-work hours.

The information collected in this study can be used by citizens to avoid mosquito bites and for control technicians planning monitoring and control programs (Table 4).

Table 4. Mosquito Activity. The best times for trapping La Crosse virus vectors in based on observed questing and oviposition activity as monitored during typical work hours (9:00-17:00) and off-work hours (17:00-9:00) using traps known to be effective at collecting LACV vectors in eastern Tennessee.

Mosquito Activity	*Aedes triseriatus*	*Aedes albopictus*	*Aedes japonicus*
Questing	Continuous	Continuous	Continuous
Ovipositing	After work	Continuous	17:00 to 9:00
Best time to collect	From 5pm to 9am	Anytime/All day	17:00 to 9:00

The best way to monitor LACV vectors is to set both questing and gravid traps at all hours and use data on timing and behavior to avoid mosquito bites. To monitor the primary LACV vector, *Ae. triseriatus,* programs should use questing traps set for 24 hours, but gravid traps can be set after work and collected in the morning. The secondary vector, *Ae. japonicus,* can be monitored using gravid traps set during off-work hours. The secondary vector, *Ae. albopictus,* should be monitored using both trapping methods set for 24 hours. This vector transmits a number of arboviruses besides LACV, including Dengue Fever virus, West Nile virus, Chikungunya, and

Zika virus, so knowing when to avoid bites and how to properly monitor and control *Ae. albopictus* has the benefit protecting citizens from all of these pathogens, not just LACV. This study confirms that all three LACV vectors are present in Knox County and previous studies have confirmed the presence of the virus as well (Jones et al. 1999, Gerhardt et al. 2001, Haddow & Odoi 2009, Urquhart et al. 2016). Knox County currently only uses a gravid trap baited with grass infused water for mosquito monitoring; specifically targeting West Nile virus and its vectors (*Culex* mosquitoes). They identify mosquitoes to genus, separate *Aedes* and *Culex* vectors into pools of 50 or fewer, and then ship samples to the State Health Department in Nashville to be screened for West Nile virus, Flanders virus, and St. Louis encephalitis. The information obtained in this study may be used to implement an effective monitoring and control program for Knox County or other areas where LACV is endemic.

Virus Screening

A novel method (RT-LAMP) was developed for the detection of LACV that takes half the time to produce results compared to the current standard method, RT-qPCR. This RT-LAMP method for detecting LACV runs at a single temperature of 58 ºC for 90 min (compared to 3 hrs by RT-qPCR) and requires equipment and reagents that cost much less than those required for RT-qPCR. The use of this method will allow for faster and more cost-effective screening of the virus from collected vectors and improve surveillance of the virus in the environment. The eastern Appalachian region is one of the few areas where LACV is endemic and while the State Health Department has the ability to screen vectors using RT-qPCR, smaller county level Health Departments do not always have the funds for a qPCR machine. Samples can be shipped to better equipped laboratories, but viral RNA can degrade en route and the shipping process delays

screening and potentially exposes citizens to the virus unknowingly because they may be less diligent about protecting themselves before understanding the extent of the danger.

The RT-LAMP method for LACV will allow counties the option to screen mosquito vectors on-site, rather than shipping them, in cases where that is currently standard. The ability to effectively and inexpensively screen for LACV at a reasonably fast pace can allow Knox County, and other counties or laboratories with limited resources, to prioritize LACV screening and surveillance. Since LACV is present in just a few areas of the United States it has not garnered the attention of more prominent pathogens like West Nile virus, Chikungunya, or Dengue fever virus (Jones et al. 1999; Haddow and Odoi 2009). The severe form of LACE is often misdiagnosed and there is currently no vaccine and no effective cure, so mosquito control and bite prevention are crucial to preventing this disease.

This newly developed RT-LAMP method will greatly increase opportunities for quick and effective monitoring of LACV prior to human infections. New equipment necessary to screen samples using RT-qPCR costs a minimum of $20,000 and the necessary reagents cost approximately $900 for 50 reactions. Conversely, an RT-LAMP can be run on a hot plate or water bath, which costs a maximum of $1,000while the reagents cost approximately $500 for the same number of reactions. Additionally, it takes three hours to complete RT-qPCR cycle and only 90 minutes to complete a RT-LAMP (Table 5).

Table 5. **Time and Pricing of Molecular Methods.** Comparison of the time and cost of equipment necessary to purchase for screening LACV by RT-qPCR and RT-LAMP, as well as the cost of reagents necessary for 50 reactions using each method and the total cost of the reagents and the equipment.

	Time	Equipment	Reagents (x50samples)	Total Cost
RT-qPCR	180 minutes	$20,000.00	$900.00	$20,900.00
RT-LAMP	90 minutes	$1,000.00	$500.00	$1,500.00

Future Directions

In order to ensure effective monitoring, it is important to continue research on the best methods for surveillance of LACV and its vectors. Changes in vector prevalence, climate, landscape, and the development of new trapping methods and lures require this ongoing effort. This study used traps known to be effective for collecting LACV vectors (Urquhart et al. 2016) and combined them with typical working hours to determine the best times and type of trap for mosquito surveillance and control in Knox County, Tennessee. Citizens can also make use of this information to know when to avoid bites and when to implement mechanical control strategies (dumping standing water, cleaning gutters) at home. Mosquitoes still questing at the end of the work day should be avoided and water should be regularly dumped in the evenings to avoid oviposition overnight. In the future, studies may be done to determine the actual nocturnal or diurnal behavior of these vectors by setting and collecting traps just before and just after sunset and sunrise or by using an automatic trap that changes nets on its own. Work hours will remain

the same, but understanding activity times more exactly may further aid citizens in knowing how and when to best protect themselves, especially those with atypical work schedules. Hourly activity measured by changing baits and collection nets or by backpack aspirator may also be worth determining to better understand exactly when mosquitoes are active and resting. Additional studies focusing on mosquito behavior may aid in the development of more effective traps and lures to further increase the number of vectors collected and controlled (Pates and Curtis 2005; Okumu et al. 2010). Research focusing on the temporal activity of questing mosquitoes attracted to the BG-Sentinel trap may provide additional insight into the behavior of *Ae. albopictus*, as well as a number of other important species. However, note that this trap is cumbersome and expensive enough to be impractical in a regular monitoring program (Farajollahi et al. 2009; Crepeau et al. 2013; Urquhart et al. 2016). Additionally, methods designed to observe other activities, such as resting behavior should be evaluated. While resting traps have not found to be effective against LACV vectors, targeting resting mosquitoes may provide useful information in the future. The use of a backpack aspirator targeting known resting places of these vectors at various times could provide this insight, as this study focused only on questing and oviposition behavior. Continuing to study trapping methods and vector behavior will contribute to the improvement of bite prevention and, as a result, disease prevention.

We need to determine sensitivity and specificity of the RT-LAMP assay and compare with the RT-qPCR. We need tests for virus specificity and non-cross reactivity to ensure no amplification occurs from other related mosquito-borne viruses. Once refined for mosquito vectors, the RT-LAMP could be tested on reservoir hosts. If it worked on reservoir hosts, it would provide further information about the presence of LACV in the environment that can be used to increase

control and prevention methods. A RT-LAMP has been developed for other pathogens, including the dengue fever virus, another important arbovirus transmitted by *Aedes* mosquitoes, as a diagnostic method. This could be an additional future possibility for LACV. While prevention is the most important tool for protecting humans, especially children, cases may still occur and the ability to more accurately diagnose patients is important. Eventually, a kit may be developed for easy purchase by laboratories that wish to use this method. This will make LACV detection much easier and more accessible to a variety of public health, research, and extension laboratories, including those without the resources to obtain expensive RT-qPCR machines and reagents. Those laboratories that do have the resources may still benefit from a less time consuming and expensive method.

Lacking an effective treatment or vaccine for LACE, we must focus on preventing the spread of the virus and the best way to do that is to prevent mosquito bites. Previous studies have provided us with information on which mosquito species transmit LACV, where they may be located, and which trapping methods are most effective for monitoring them. However, previous studies have also not considered the typical work day as a potential factor in how professional mosquito monitoring and control programs should be implemented nor in considerations of public advice. Additionally, the gold standard for virus detection is still out of practical reach for many laboratories thus slowing down virus surveillance and subsequent public warnings. In this project, we determine the best monitoring times for LACV vectors as exploitable by mosquito control professionals working a typical 9:00-17:00 work day. This information is also crucial to helping most citizens understand how and when to best protect themselves in accordance with their own schedules. We have also designed a novel molecular method for the detection of

LACV from mosquitoes, which can be used to determine high risk areas more quickly than in the past. This gives us the tools necessary to provide the public with the information they need to protect themselves earlier and more effectively than in the past. These improvements in surveillance, control, and bite prevention will improve public health in LACV endemic regions and help prevent diseases.

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APPENDICES

APPENDIX 1

RNA Extraction Protocol

Reagents

- Chloroform (Life Technologies: Catalog #15593031)
- Trizol reagent (Life Technologies: Catalog #15596-18)
- 100% Isopropanol (Fisher Scientific: Catalog #A416-500)
- 75% ethanol diluted from 95% (Sigma-Aldrich: Catalog #493511)
- RNase-free water (Qiagen: Catalog #129112)

Equipment

- Refrigerated centrifuge capable of reaching 12,000 x g
- RNase/DNase-free microcentrifuge tubes
- Water bath (55-60ºC)
- Tungsten carbide beads

Steps

1. Homogenization
    a. Add 200µl of trizol reagent to mosquito pool, mosquitoes should be in a RNase/DNase free 1.5ml tube
    b. Homogenize with power homogenizer or add 3 beads to tube and vortex for 10-15 seconds to break-up mosquito tissue
    c. Homogenized samples can be stored at -80ºC for one month
        i. Date the box and vial with date homogenized for tracking
2. Phase Separation
    a. Incubate homogenized samples for 5 minutes at room temperature
    b. Add 50µl of chloroform to homogenized sample and secure cap on tube
    c. Shake tube vigorously by hand for 15 seconds. Do not vortex.
    d. Incubate at room temperature for 2-3 minutes
    e. Centrifuge at 12,000 x g for 15 minutes at 4ºC
        i. Note: There will now be 3 noticeable layers in the tube; a red phenol-chloroform phase on the bottom, an opaque white interphase in the middle, and a clear aqueous RNA phase at the top. The RNA is contained in the clear aqueous phase.
    f. Pipette out the aqueous phase, being careful not to remove any of the other layers. It may help to hold the tube at a 45º angle.
    g. Transfer the aqueous phase to a new, pre-labeled, 1.5 ml RNase/DNase free tube
3. RNA Precipitation
    a. Add 100µl of 100% isopropanol to the RNA tube
    b. Incubate at room temperature for 10 minutes
    c. Centrifuge at 12,000 x g for 10 minutes at 4ºC (refrigerated centrifuge)
        i. Note: The RNA may form a visible pellet after centrifuging, but it may also be invisible.
4. RNA Wash
    a. Remove the supernatant from the tube, leave only the RNA pellet

        i. Note: If the pellet is invisible, be careful to pipette the supernatant only from the bottom or from the side of the tube that was facing the inside of the centrifuge during step 3c.
    b. Wash the pellet with 200µl of 75% ethanol by adding it to the tube
        i. Note: The sample can be stored at -20ºC for at least a year at this point.
    c. Vortex the sample for 2-3 seconds, then centrifuge at 7,500 x g for 5 minutes at 4ºC. Discard the wash.
    d. Air dry RNA pellet for 5-10 minutes by leaving the lid open in a safe place from contamination (ex. hood)
        i. Note: Do not allow pellet to dry completely as it may lose solubility.
5. RNA Resuspension
    a. Re-suspend RNA pellet in 20-50µl RNase-free water by passing the solution up and down several times through a pipette tip
    b. Incubate in water bath at 56ºC for 10-15 minutes
    c. Store at -80ºC

APPENDIX 2

RT-qPCR Protocol

Reagents

- Quantitect Probe RT-PCR Kit (Qiagen: Catalog #204443)
- TaqMan Probes (Life Technologies: Catalog #4316034)

Materials

- 96well plate
- Quantitative PCR machine

Steps

Before working with reagents

1. Turn on PCR hood light and fan
2. Clean PCR hood with 10% bleach followed by 70% etoh
    i. This is important b/c reagents can easily be contaminated
3. Ready an ice bucket or tray
4. Remove reagents and samples from freezer and allow to thaw (15-20 minutes)
    i. Do not go longer or samples will degrade
5. Once thawed, place reagents and samples on ice to keep samples from degrading

Mastermix

6. Create mix with the following volumes and concentrations of each reagent per well: KEEP ON ICE!
    i. 12.5µl Master Mix (from kit)
    ii. 0.25µl Forward Primer
    iii. 0.25µl Reverse Primer
    iv. 0.15µl Probes
    v. 0.25µl RT Mix (from kit)
    vi. 6.6µl H_20 (from kit)
7. Return reagents to freezer (-80C) to keep the integrity of the product

RT-qPCR assay

8. Add 20µl mastermix to each well (from step 6)
9. Add 5µl sample to each well, & be sure to include a 5µl positive and 5µl negative control in each row or column (depending on how you set up the reaction)
10. Spin plate using a plate spinner or centrifuge for 10-20 seconds to ensure samples and mastermix are all at the bottoms of the wells (if a spinner is unavailable, be careful to pipette sample into the bottom of the well and tap the tray down to get any drops off the sides of the wells)
11. Return samples and reagents to -80ºC freezer
12. Re-clean PCR hood, turn off fan and light

13. Place plate in qPCR machine and set reaction conditions
    i. Cycle 1:
        1. Step 1: 40min at 50ºC
        2. Step 2: 15min at 95ºC
    ii. Cycle 2: (45X)
        1. Step 1: 0:20min at 94ºC
        2. Step 2: 1min at 60ºC
        3. Step 3: 0:15min at 62ºC
    iii. Cycle 3
        1. Step 1: 0:15min at 72ºC
    iv. Cycle 4
        1. Hold at 4ºC
14. Observe results on graph
    i. Curves above the threshold level are positive

Other

For further confirmation of results, positive samples may be visualized via gel electrophoresis Samples may also be sequenced

APPENDIX 3

RT-LAMP Protocol

Reagents

- avian myeloblastosis (AMV) (Invitrogen, Life Technologies: Catalog #12328019)
- *Bacillus stearothermopolis* DNA polymerase (*Bst*) (New England BioLabs Inc.: Catalog #M0275L)
- Betaine (Sigma Aldrich: Catalog #B0300)
- dNTPs (Life Technologies: Catalog #18427013)
- Thermopol buffer (New England BioLabs, Inc.: Catalog # M0275L)
- Primers
- $MgSO_4$ (New England BioLabs, Inc.: Catalog # M0275L)
- RNase/DNase free H_2O

Materials

- PCR Strips
    - Using 96well plates is NOT a good idea b/c it can get difficult to see turbidity
- Thermalcycler or Water bath (60ºC)

Steps

Before working with reagents

15. Turn on PCR hood light and fan
16. Clean PCR hood with 10% bleach followed by 70% etoh
    i. This is important b/c reagents can easily be contaminated
17. Ready an ice bucket or tray
18. Remove reagents and samples from freezer and allow to thaw (15-20 minutes)
    i. Do not go longer or samples will degrade
19. Once thawed, place reagents and samples on ice to keep samples from degrading

Mastermix

20. Create mix with the following volumes and concentrations of each reagent per well: KEEP ON ICE!
    - 1.01µl of 15U/µl AMV
    - 1.32µl of 8U *Bst*
    - 3µl of 5M betaine
    - 6.25µl 20mM dNTPs
    - 2.5µl 10X Thermopol Buffer
    - 0.5µl 100µM FIP and BIP (primers)
    - 0.05µl 100µM F3 and B3 (primers)
    - 0.25µl 100µM FLoop and BLoop (primers)
    - 3.32µl $MgSO_4$
    - 1µl H_2O

21. Return reagents to freezer (-80C) to keep the integrity of the product

RT-LAMP assay

22. Add 5µl sample to each well, & be sure to include a 5µl positive and 5µl negative control (If you have a strip of 8 wells, 2 wells should be used for controls and 6 for samples)
    i. Return samples to freezer (-80C) to keep the integrity
23. Denature all of the samples in the strip by heating the strip to 95ºC for 60 seconds; you are breaking up the sample so mix is easy to get to
24. Add 20µl mastermix to each well (from step 6),
    i. Put the mix at the bottom of each well and pipette up and down several times
    ii. Between each well (sample) change the tip(!!!) to avoid contamination
    iii. Each well should now have 25µl (5µl of sample + 20µl of mix)
25. Heat wells containing mastermix and samples at 58ºC for 90 minutes
26. Shake wells to check for turbidity,
    i. Record turbid samples; note you may have to pipette each sample a bit or use vortex for split second; if you pipette change tips
27. Return samples and reagents to freezer (-80C)
28. Re-clean PCR hood, turn off fan and light
29. Observe samples for turbidity (if turbidity is not detected, vortex samples for a fraction of a second and re-check

Other

RT-LAMP samples can be verified in a gel, they cannot be sequenced
Positive RT-LAMP samples can be used as a positive control if diluted with water
Negative RT-LAMP samples can be used as a negative control if diluted with water

VITA

Cassandra Urquhart is originally from Bedford, Massachusetts where she began exploring her interest in entomology at an early age. She attended Sonoma State University in Rohnert Park, California and later transferred to the University of Massachusetts in Amherst, Massachusetts where she received a Bachelor's Degree in Natural Resource Studies with a minor in entomology. Cassandra worked in entomology laboratories throughout her undergraduate career and was a volunteer in a Harvard Biomechanics laboratory following graduation. She was offered a medical entomology internship with the Tennessee State Department of Health in 2013. From there became a graduate student at the University of Tennessee, Knoxville where she is currently finishing her Master's Degree in Entomology and Plant Pathology with a concentration in Medical and Veterinary Entomology.