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I am submitting herewith a dissertation written by Andrew Douglas Haddow entitled “The Epidemiology of La Crosse Virus in the Tennessee and West Virginia.” I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Plants, Soils, and Insects.

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The Epidemiology of La Crosse Virus in Tennessee and West Virginia

A Dissertation Presented
for the Degree of
Doctor of Philosophy
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Andrew Douglas Haddow
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For My Parents

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ABSTRACT

La Crosse virus (LACV) was first recognized as a cause of pediatric viral encephalitis in the upper-Midwestern United States following its isolation from a fatal case of pediatric encephalitis in 1964. From that time, LACV infections have been reported in 24 states, with the number of reported cases increasing in recent years in the Appalachian region of the United States. Two states in Appalachia, West Virginia and Tennessee have both seen a large rise in the number of reported cases in recent years.

To investigate the epidemiology of the LACV infections in the eastern United States, Tennessee, and West Virginia a combination of field and laboratory studies were initiated. These studies investigated the spatial and temporal patterns of disease risk and the biology and ecosystem dynamics of both indigenous and invasive disease vectors.

Four distinct regional clusters of LACV infections were detected at the national level, as well as a higher incidence risk and case-fatality rate than previously reported. The exploration into the variations of risk using different at-risk populations and geographic scales revealed the possibility of missing disease clusters resulting from performing incidence risk investigations of focal diseases using inappropriate at-risk populations and/or large geographic scales. South-central West Virginia was found to be a focus of LACV transmission and in addition to having the highest incidence risk and case-fatality rate reported in the United States.

In eastern Tennessee, *Aedes albopictus* was found to be the most abundant mosquito collected at all sites and vegetation types, by both CO₂-baited CDC trapping and human landing catches. Results from the use of variable pressure scanning electron

microscopy to describe the egg of *Aedes japonicus* have provided more detailed information on characters of Aedini eggs, while bringing the number of more complete descriptions and micrographs of the micropyle and associated structures of the subgenus *Finlaya* to three.

The results of these studies have provided a more complete understanding of the epidemiology of LACV and its associated vector species at both the national and state levels. These findings will help guide future research and intervention efforts to understand and prevent virus transmission.

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Preface

La Crosse virus (LACV) was first isolated from the brain of a deceased pediatric patient who died of encephalitis in 1964 [1], and is one of the most common causes of pediatric arboviral encephalitis in the United States [2,3]. The traditional focus of virus transmission has been in the upper-Midwestern United States [2,4,5], but more recently LACV has been seen as an emerging disease in Tennessee and West Virginia [6,7,8,9]. Following the initial outbreaks of infections in 1987 and 1997, LACV infections have continued to rise in both Tennessee and West Virginia respectively [6,10]. This project set out to (1) determine the current spatial and temporal patterns of disease risk for LACV first at the national level, and then within Tennessee and West Virginia, and (2) to determine the current vector ecosystem dynamics in eastern Tennessee.

Prior to this project the spatial epidemiology of LACV at the national and state levels were not well understood, and only one study of the spatial clustering and risk factors of LACV had been reported in the literature [11], though foci were known to exist in several states [4,5,12,13,14]. Additionally, reported incidence risks for LACV were limited [10,15]. Therefore, this project conducted spatial and temporal analyses of LACV infections and to determine those areas of highest risk both nationally and at the census tract level for Tennessee and West Virginia.

The effect that the indigenous vector *Aedes triseriatus* and two invasive mosquito vectors, *Aedes albopictus* and *Ae. japonicus*, have on the sylvatic and urban transmission cycles of LACV in eastern Tennessee is unknown, though previous studies in Tennessee and West Virginia both have shown that both invasive species could be playing roles as accessory LACV vectors [16,17,18,19]. The role of *Ae. triseriatus* in LACV

transmission is well understood [20,21,22], and though *Ae. albopictus* and *Ae. japonicus* have been shown to be competent laboratory vectors [17,23,24], the role of environmental and habitat characteristics affecting these species vectorial status in eastern Tennessee remained in question. To further the understanding of the ecology of vector species and the ecosystem dynamics surrounding LACV transmission, field studies were undertaken in eastern Tennessee from 2004 to 2006. To provide more detailed information on characters of Aedini eggs, and in an effort to assist field biologists in the identification of predominantly collected tree-hole species/container species, the egg of *Ae. japonicus* was described with the aid of the variable pressure scanning electron microscope.

SECTION I: BACKGROUND AND LITERATURE REVIEW

CHAPTER I

Background

Introduction

La Crosse virus (LACV) is a member of the genus *Orthobunyavirus*, family Bunyaviridae, and is the causative agent of LACV infections. LACV was first isolated in 1964 [1], and since that time has become one of most common causes of pediatric arboviral infections in the United States [2,3]. LACV has traditionally been associated with forested areas in the upper-midwestern United States [4], but more recently as an emerging disease in the Appalachian region of the United States [6,8,9]. The majority of LACV infections are transmitted to humans through the bite of the primary vector, the eastern tree-hole mosquito, *Aedes triseriatus* [20,25]. The virus is maintained in nature through a vertical and horizontal transmission cycle involving *Ae. triseriatus* [21,22,26] and the primary amplification hosts: the eastern chipmunk, *Tamias striatus*, the gray squirrel, *Sciurus carolinensis*, and the fox squirrel *Sciurus niger* [27,28].

Since its recognition as a cause of human illness, LACV infections have been reported in 24 states [29], with the number of reported cases increasing in recent years in the Appalachian region of the United States [6,8,9,10]. Two states in Appalachia, West Virginia and Tennessee have both seen a large rise in the number of reported cases in recent years following initial large outbreaks of infections in 1987 and in 1997, respectively [6,10]. Following these outbreaks the virus became a seasonally recognized cause of viral encephalitis in these States [6,16,30].

Risk Factors

Following the initial outbreak in West Virginia, a case control study was undertaken to determine the effects tree holes, artificial containers, and environmental and behavioral characteristics have upon the transmission of LACV in West Virginia [30]. A slight increase in risk ($OR < 2.0$) was found for an increased time spent outdoors, the non-use of insect repellent, the non-use of air conditioning, a lack of screened windows, and the non-use of protective clothing. The presence of tree holes near a residence was found to significantly increase the risk of transmission ($OR = 8.5$ for ≥ 1 tree hole vs. 0 tree holes).

Factors that were found to not statistically significantly increase the risk of disease transmission were artificial containers ($OR = 4.1$ for ≥ 6 containers vs. 0 to 5 containers), discarded tires ($OR = 3.2$ for ≥ 10 tires vs. 0 to 9 tires), and the proximity to forest edge ($OR = 3.2$ for 0 to 14.9 m vs. ≥ 14.9 m). The authors concluded that though these factors were not statistically significant, they could still result in increased disease transmission not detected due to limitations of the study. This study took place during a period of low rainfall, and authors hypothesized that tree holes would hold larger amounts of water than artificial containers. Therefore, tree holes would provide more larval habitat than artificial containers during these periods of time, biasing the results. The matching of controls by social and economic characters may have limited the study's ability to detect differences between study households, i.e., the number of tires in proximity to households.

Further limitations of this study identified by the authors included the lack of larvae collected and identified near residences due to the time of year (autumn), the inclusion of a study area around the residence of 91.4 m, though children would have spent a larger proportion of time beyond this radius. The authors concluded that these results could have under or over-estimated disease transmission in the peridomestic environment.

In 2002, the first blinded case control study looking at the clinical, environmental, and the entomological characteristics of LACV was carried out in eastern Tennessee [16]. Significant factors found to be associated with LACV infection were found to be the number of hours per day spent outdoors (5.9 for LACE cases vs. 4.0 for non-cases, $p = 0.05$) and living in a residence with one or more tree holes within a 100 m radius from the dwelling (relative risk of 3.96 vs. no tree holes in a 100bm radius, $p = 0.05$). No correlation was made with the type of clothing worn, the use of insect repellant, or the use of screens on open windows.

Vector Surveillance

In Nicholas County, West Virginia, Nasci et al. [9] conducted a study to associate habitat with the transmission of LACV in 1996, prior to the establishment of *Ae. albopictus* in West Virginia. Sites were characterized by habitat type, distance from reported case sites of LACV infections, and included both host-seeking and ovitrapping mosquito collections. LACV-infected *Ae. triseriatus* species were found at all study sites. *Aedes triseriatus* eggs were used a measure of population density and showed an increase in population from the beginning of the season through mid-July, from mid-July

through early-September populations plateaued, with population densities greatest (38 percent to 46 percent) at collection sites adjacent to reported cases of LACV infections. The seasonal total LACV infection rates in transovarially infected mosquitoes was found to range from 0.4/1,000 to 7.5/1,000 and were not found to be statistically significant by either habitat characterization or distance to reported LACV infection case sites. The season total LACV minimum infection rates ranged from 4.3/1,000 to 27/1,000 in host-seeking *Ae. triseriatus* species collected and from 1.6/1,000 to 2.4/1,000 for host-seeking *Ae. canadensis* an accessory vector of LACV [31,32]. There was no significant difference found between habitat type and distance to reported cases of LAC encephalitis and the presence of infected mosquitoes.

Starting in 1992 and continuing through 2003, the results from a series of studies [18,19,33,34] inferred the distribution and seasonality of mosquito vectors in West Virginia. The first of these studies found that populations of *Ae. triseriatus* were distributed throughout the state and at all elevations sampled [34]. *Aedes triseriatus* larvae were found to be significantly ($p < 0.05$) associated with shaded habitats.

Following this study the presence of vector species was investigated at waste tire dumps in Nicholas County [18]. The authors found that *Ae. triseriatus* was the most abundant species collected, and it was significantly more likely ($p < 0.05$) to be collected in forested habitat, while *Ae. albopictus* larvae were significantly ($p < 0.05$) more likely to be collected in peridomestic areas.

In 2002, Joy et al. [33] observed that *Ae. triseriatus* was the most abundant species collected from abandoned tire pile sites, except during September when *Ae. albopictus* and *Aedes japonicus* were found to be more abundant. It appears that during

this time period between 2001 and 2002, *Ae. albopictus* and *Ae. japonicus* became firmly established in West Virginia and their respective populations increased.

In a 2003, a follow-up study conducted to determine the occurrence of mosquito larvae inhabiting tires in different geographic regions (western, central, and eastern) of the state [19] provides the most recent account of the biology and distribution of vector species in West Virginia. *Aedes japonicus* was found to be the most commonly collected species in the state in all geographical regions. Populations of *Ae. japonicus* remained roughly constant throughout the season, except for lower collections made in March and April in the western region of the state. *Aedes japonicus* was significantly more abundant ($p < 0.05$) than *Ae. triseriatus* in the eastern region of the state and in peridomestic areas in all regions, though both species were found to be collected at relative numbers in non-peridomestic areas.

Aedes triseriatus was the second most abundant species collected in West Virginia. In agreement with previous work [34], populations peaked roughly in August and declined into October. *Aedes albopictus* collected experienced low numbers from April through June, with numbers gradually increasing until October. It was most frequently collected in the western region of the state, with the central region have fewer collections, and the eastern region having the lowest number of collections. Populations were similar at both peridomestic and non-peridomestic sites. Numbers of *Ae. triseriatus* were found to be significantly greater ($p < 0.05$) than collections of *Ae. albopictus* in both peridomestic and non-peridomestic sites in the central and eastern regions of the state.

This study inferred a shift in vector abundance with *Ae. japonicus* being collected more frequently than *Ae. triseriatus* in all geographic regions of West Virginia, thus

increasing the likelihood that this species could be an accessory vector of LACV in West Virginia. The findings that *Ae. albopictus* were collected at significantly lower ($p < 0.05$) numbers than *Ae. triseriatus* at both peridomestic and non-peridomestic sites in the central and eastern regions of the state could have implications for its role as an accessory vector of LACV in these regions [9,10,30], though virus isolation studies are needed to confirm this hypothesis.

Similar studies were carried out in Tennessee following the initial LACV outbreak in 1997. These studies took place following the establishment of *Ae. albopictus*. In order to assess the temporal patterns, parity, survival rates and vector potential in both *Ae. albopictus* and *Ae. triseriatus* surveillance was undertaken at two case sites of LACV infections that occurred in 1997 [35]. This study showed that the monthly mean parity rates for *Ae. albopictus* from July through October ranged from 0.78 to 0.92, and did not differ significantly ($p > 0.05$) between the two LACV infection sites. The monthly mean parity rates for *Ae. triseriatus* adults ranged from 0.71 to 0.80 during the study period, but low numbers of collected *Ae. triseriatus* prevented statistical comparison with parous *Ae. albopictus*. The estimated survival rate of adult *Ae. albopictus* females ranged from 0.92 to 0.99 during this period. The high parity and survival of *Ae. albopictus* adults indicated that a large percentage of the population was multi-parous, thus increasing the chances of becoming infected and transmitting LACV.

The first isolate of LACV in Tennessee, came from *Ae. albopictus* (TN00-2266) resulting from follow-up vector surveillance at a LACV infection case site in 1999 [17]. The minimum infection rate for *Ae. albopictus* eggs oviposited at the site was determined to be 6.5/1000 for the isolation week, with a ratio of collected *Ae. albopictus* to *Ae.*

triseriatus of 153:1. This residence was well maintained within an oak and hickory forest located 15 meters from the residence. A later study [16], found that the burden of *Ae. albopictus* collected (three times greater at cases than non-cases, $p = 0.013$) was a significant risk factor associated with LACV infection. The results of this study suggested that *Ae. albopictus* might be playing a greater role in LACV transmission in eastern Tennessee than elsewhere, e.g. Wisconsin. No statistical difference was found in the burden of *Ae. triseriatus* around residences of LACV cases and non-cases.

Prevalence

To determine the prevalence of LACV in eastern Tennessee, serum samples originally collected from Knoxville and the surrounding 15 counties to test for *Treponema pallidum*, HIV, and rubella virus were tested for LACV in 1999 [7]. Serum samples from 1000 patients were screened for the presence of IgG antibodies, with a cutoff of 4. Those specimens with a final titer of ≥ 8 were considered positive. Results showed a prevalence of 0.5 percent in the samples tested. The authors concluded that due to the low prevalence, LACV was most likely newly endemic to Tennessee, though this conclusion should be interpreted with caution as sample specimens came from a biased group. The true extent of LACV prevalence in eastern Tennessee is unknown.

Early work in the Cherokee Indian Reservation in North Carolina found a prevalence rate of LACV in high school students of 11.3 percent [36]. Recent work by Szumlas et al. [37] has shown a prevalence of up to 20.6 percent on the Cherokee Indian Reservation. Samples taken in areas outside the reservation indicated a prevalence of only 4.7 percent, and is higher than the 0.5 percent prevalence in eastern Tennessee. No

serosurvey has been conducted in West Virginia to date, but it is likely that prevalence in the population is similar or higher to that of North Carolina due to the large number of reported cases.

Surveillance for LACV was expanded in eastern Tennessee in 2003 to include the trapping and testing of host species for the presence of LACV neutralizing antibodies [38,39]. These studies were conducted on populations of the eastern grey squirrel, *Sciurus carolinensis*, and the eastern chipmunk, *Tamias striatus*, at two sites (A and B) in eastern Tennessee from 2003 to 2005. Site A had two cases of human LACV infections previously reported, and was located in Knox County, Tennessee. Site B had no previously reported cases of LACV infections, and was located in Blount County, Tennessee. Four *S. carolinensis* and two *T. striatus* collected at site A, had detectable levels of LACV neutralizing antibodies during the study period, accounting for a prevalence of 2.65 percent. A single *T. striatus* collected every year of study period at site A, had demonstrated detectable LACV neutralizing antibodies for 717 days, the longest recorded persistence of LACV antibodies [28,39,40]. One *S. carolinensis* collected at site B during the last collection week of 2005, had detectable levels of LACV neutralizing antibodies, accounting for a prevalence during the study period of 0.44 percent. The detection of neutralizing antibodies to LACV in the *T. striatus* collected at site B during the last collection in 2005 could be an indication of recent introduction of LACV to this site, and highlights the possibility of virus expansion into areas previously not associated with the virus.

Clinical Studies

Studies in both states provide the most current picture of LACV infections [6,8,16,41] and provide clues to the severity and course of the illness. The majority of LACV infections in Tennessee and West Virginia occur in the summer months, in males, and in children 15 years and younger [10,16,30,41], these finding agree with reports from previous studies elsewhere [2,5,42].

The most complete description of the clinical course of LACV infections came from a retrospective study of 127 patients with confirmed cases of LACV infections from 1987 to 1996 in West Virginia [8]. On admission, both vomiting ($p = 0.05$) and a Glasgow Coma Scale score of 12 or lower ($p = 0.05$), were found to be significantly higher for patients with LACV infections than other patients. LACV patients experienced a variety of symptoms: 70 percent experienced headache, fever, and vomiting; 46 percent suffered seizures, 42 percent experienced disorientation, 21 percent developed hyponatremia, and 13 percent developed increased intracranial pressure. Decreases in serum sodium levels ($p = 0.01$) and increased body temperature ($p = 0.01$) indicated deterioration. Neurological deficits were recorded in 12 percent of patients at the time of discharge, and follow-up assessments showed cognitive and behavioral deficits 10 to 18 months post infection.

Results from the Tennessee case control study [16], found no statistically significant ($p > 0.05$) differences in the presence of fever, headache, vomiting, or behavioral changes between the two groups. Though a later study in Tennessee [41], found that LACV patients were significantly more likely than enteroviral central nervous

system (EV-CNS) infections to have aphasia ($p = 0.001$), loss of consciousness ($p = 0.001$), seizure ($p = 0.001$), and admission to a pediatric intensive care unit (ICU) ($p = 0.05$).

Though most infections are asymptomatic or result in a relatively mild febrile illness in humans, in a sub-group of patients' disease progresses to severe meningitis, encephalitis, or meningoencephalitis [8,43,44,45]. Following the course of illness variety of sequelae have been reported in the literature [8,45,46].

Summary

The Appalachian region of the United States has shown significant increase in the reported cases of LACV infections in the last 20 years (CDC unpublished), with both Tennessee and West Virginia experiencing a large increase in the number of reported infections in recent years. Several previous studies in both states have provided insight into the risk factors, vector species, prevalence, and the clinical presentation of LACV infections, but several questions remain: are there areas of significant disease clustering; what is the overall risk of infection in these states; have the risk factors for infection changed from previous studies; what are the current vector ecosystem dynamics at case and non-case sites of LACV, what is the status of non-native vector species?

CHAPTER II

Literature Review

Virus

La Crosse virus (LACV) belongs to the family *Bunyaviridae*, genus *Orthobunyavirus*, and is a member of the California serogroup of viruses. The viron is approximately 90-100 nm in diameter. The genome of LACV is tri-segmented being comprised of single-stranded RNA segments of negative polarity grouped according to size: large (L), medium (M), and small (S) [47]. These segments form helical ribonucleoprotein complexes (RNPs) within the nucleocapsid (N) protein. The L segment encodes for the viral polymerase the source of transcription and the replication of the genome [48,49]. The M segment encodes the Gc (formerly G1) and Gn (formerly G2) surface transmembrane glycoproteins [50] and NSm (function unknown) through a single open reading frame. Gc and Gn appear to serve as a heteromultimer following cleavage of the polyprotein [51]. Following the location by Gn this heteromultimer targets the Golgi apparatus; the location of both viral assembly and budding [51,52,53]. Gc is the viral attachment protein, serves as the binding site of the neutralizing antibodies, and mediates pH-dependent fusion serving as a class II fusion protein and is likely serves as a fusion peptide [54,55,56,57] [53,58,59,60]. The S segment encodes for the viral nucleocapsid (N) protein and the non-structural protein NSs [61,62,63]. The N protein in turn encapsulates the RNA segments and the non-structural protein NSs which suppresses

RNA inference, playing a role in invertebrate antiviral defense [64,65], and may have evolved to suppress the mammalian innate immune response [66].

Genotypes

Three isolates of LACV have been made in humans from post mortem brain tissue; Minnesota in 1960, Wisconsin in 1978, and Missouri in 1993 [67]. The remaining isolates all originated from mosquito species. Huang et al. [67] demonstrated that fatal cases of severe LACV infections may come from a narrow range of highly conserved genotypes. Previous studies using DNA fingerprinting of LACV isolates grouped LACV strains into three genetic groups A, B, and C [68,69], and recent data suggests that there are three distinct lineages of LACV [70]. Groups A, B, and C corresponding to lineages 1 and 2, with lineage 3 being a distinct group following analysis of the Connecticut isolate (6716-05) [70]. It is unknown if these lineages exhibit differences in virulence, infectivity, and host specificity.

Virus Evolution

Evolution of LACV most likely occurs through antigenic drift and antigenic shift (RNA segment reassortment), both of which are common during persistent infections [71]. Antigenic drift has been demonstrated in LACV with no two isolates sharing oligonucleotide fingerprints [68,69], and is likely the main mechanism for evolutionary change within the virus. The nature of transovarial transmission (TOT) within infected vectors would increase the chances of genetic diversity within vector populations as

reassortment could occur in the infected ovaries following infections of secondary viruses [72].

Antigenic shift has been demonstrated in vitro, vivo, and in nature [71,73], and only viruses within the serogroup appear capable of reassortment [74,75]. Both the vector and vertebrate hosts could serve as sites of possible reassortment in nature [72], though reassortment has not been demonstrated in animal models and its rate in nature is unknown [71,74]. Within the vector populations' reassortment has been shown to occur at a high level. Reassortment occurs when the vector becomes infected with a second virus within one to two days post infection of the initial virus; by day three the vector becomes refractory to superinfection [74]. Interrupted feeding would increase the chance that a vector could become infected with several viruses [71]. During this practice the vector would take a bloodmeal from several different hosts in a relatively short period of time. Providing that the hosts were viremic with different viruses of the same serogroup, reassortment could occur.

Reassortment is also likely to occur through TOT. Transovarially infected (TI+) mosquitoes have been shown to experience lower virus titers in all tissues than those mosquitoes infected through the oral route [72]. Borucki et al. [76], found that 20 percent of mosquitoes that were TI+ became superinfected following ingestion of bloodmeals containing wild-type LACV or Snow Shoe Hare virus (SSHV). Of the genomes tested: 2.3 percent experienced reassortment, and 4.0 percent were shown to be heterodiploid. As the females mosquitoes are infected for life each additional bloodmeal would increase that chances of reassortment occurring in the ovaries, whereupon the new genotypes would be passed onto progeny.

Virus/Vector Relationship

After ingestion of an infective blood-meal LACV infects and replicates in the epithelial cells of the culicine midgut [25,77]. The virus escapes the midgut whereupon it infects the hemocoel; it then infects and replicates the neural ganglia, heart, fat bodies, ovaries and finally the salivary glands between day 7 to 16 [72,77]. The virus reaches high titers in the salivary glands and is passed to the host during the act of feeding. Infection and infectiveness of the vector is life long.

LACV infects ovaries by which it is passed by to the progeny [78,79]. Both male and female progeny are infected through this process; infected males are then able to infect uninfected females through venereal transmission via accessory sex fluid gland fluid [26]. The virus overwinters via TOT [71,80], which appears to cause no negative effects on the oocyte and/or the embryo [77], demonstrating a unique co-evolution between the vector and virus.

Virus Isolations from Invertebrates

LACV has been isolated from the mosquito species *Ae. triseriatus* [20,81], *Ae. albopictus* [17], *Ae. canadensis* [31], *Ae. communis* [82], *An. punctipennis* [12], *Ae. sollicitans* [12], *Ae. trivittatus* [12], *Ae. vexans* [12], *Coquillettidia perturbans* [12], and *Culex pipiens* [83]. Additionally, LACV has been isolated from the horse fly *Hybomitra lasiophthalma* [84]. *Aedes japonicus* has been shown to be a competent laboratory vector of LACV [24].

Prototype Vector

Historically, *Ae. triseriatus*, a species native to North America, has been the primary vector of LACV for much of the United States [2,21]. LACV can be transmitted transovarially and venereally in this species and is able to overwinter in eggs [4,21,22,26,85]. It is primarily a tree-hole mosquito, but will also oviposit in man-made containers. It is known to feed on a variety of mammals including deer, squirrels, chipmunks and humans [86]. *Aedes triseriatus* is widely distributed throughout the eastern United States [87] and has been found at LACV case sites in eastern Tennessee [16]. *Aedes triseriatus* likelihood of involvement in the horizontal transmission cycle, coupled with its higher midgut infection and dissemination rates of LACV through oral infection [88], make it the likely primary vector of LACV in the eastern United States.

Accessory Vectors

Aedes canadensis is considered an accessory vector of LACV [9,12,31]. Though infection rates similar to those found in *Ae. triseriatus* have been found in both Ohio [12] and West Virginia [9], it remains less efficient at transmitting the virus than *Ae. triseriatus* [32]. *Aedes canadensis* is typically thought to have one generation per year in northern areas, though second generations have been reported in Ohio [31]. Warmer temperatures in the southern United States would increase the likelihood of several generations occurring in one season and thus increasing its involvement in LACV transmission.

Aedes albopictus, an invasive species, is considered to be a probable accessory vector of LACV, following laboratory and field studies [16,17,23]. Larvae of *Ae.*

albopictus were discovered in Houston, Texas in 1985 [89] and is believed to have been introduced to the United States through shipments of tires from Asia [90,91]. Since its establishment, *Ae. albopictus* has spread over a large portion of the United States [92]. Its larvae are found in both man-made containers and tree holes. *Aedes albopictus* is known to feed on a variety of animals, including humans [93]. LACV has been isolated from *Ae. albopictus* in eastern Tennessee [17]. It is probable that *Ae. albopictus* serves as an accessory vector, and that its high abundance and aggressive biting may overcome its shortcomings in its potential to acquire, develop, and transmit LACV.

Aedes japonicus, another invasive species, was recently introduced into the United States from Asia [94]. Since its introduction into the United States in 1999 [95,96], it has spread rapidly across the United States. *Aedes japonicus* has been shown to be a competent laboratory vector of LACV [24], and though this species has established in LACV endemic regions [95,97,98,99], its current role in the maintenance and transmission of LACV remains unknown. To date there are no reported isolations of LACV from this species.

It is unknown what the overall role several culicid [12,82,83] and one tabanid species [84] play in the transmission and maintenance of LACV, though due to the low number of virus isolations, host-feeding preferences, and abundance at LACV foci, it is likely that these species play little or no role in virus transmission.

Virus/Host Relationship

LACV has been isolated and studied in several species [28,100,101,102,103], but the preferred animal model for studying LACV pathogenesis remains suckling mice

[104]. LACV isolates inoculated subcutaneously in mice have been found first replicate muscle tissue with viremia developing, whereupon the virus crosses the vascular endothelial cells and invades the brain [104,105,106,107]. Neurons have been shown to be the predominately-infected cells in the CNS [66,104]. As in humans, pediatric mice are more susceptible to severe LACV infections than adults [104]. Following, the finding that LACV can infect via the nasal route [103], the authors reiterated the hypothesis that exposure to water containing LACV infected larvae or free virus from lysed larvae, could pose an alternate route nasal/oral for transmission of the virus to host species [108]. This hypothesis remains to be tested but would have implications to virus maintenance and amplification if found to be a viable alternate route of transmission.

Hosts

LACV maintenance is assisted in a horizontal transmission cycle involving the primary vector *Ae. triseriatus* and the primary amplification hosts the eastern chipmunk *Tamias striatus*, the gray squirrel, *Sciurus carolinensis*, and the fox squirrel, *Sciurus niger* [4,25,27,28]. These species show no clinical manifestations associated with infection and are viremic for 1 to 2 days [109,110]. Infection provides them with lifelong immunity to the virus [111].

Secondary hosts, including the red fox, *Vulpes fulva*, and the gray fox, *Urocyon cinereroargenteus* [112], may spread LACV to non-endemic foci through movement into new areas. The deer mouse, *Peromyscus maniculatus*, and the white-footed mouse, *P. leucopus*, do not experience significant viremias [112,113] and similarly domestic animals have not been shown to become viremic post exposure [4].

Aedes triseriatus is known to feed on a variety mammals including deer, squirrels, chipmunks, and humans [86,114], while *Ae. albopictus* is considered a catholic feeder and is known to feed on a diverse range of mammalian, avian, and reptilian species [93,114,115]. The differences in feeding behaviors between these two species coupled with differences in vegetation/habitat preferences would make it more likely that a higher proportion of *Ae. triseriatus* would come in contact and to feed on the primary amplification hosts, *Tamias striatus*, *Sciurus carolinensis*, and *Sciurus niger* [4,25,27,28].

Geographic Distribution, Incidence, and Environmental Risk Factors

LACV infections have been reported in 28 states in the eastern United States [70] since its recognition in 1964 as a cause of human illness [116], and has traditionally been associated with forested areas in the upper-midwestern United States [4,5,117]. There have been an average of 79 nationally reported cases of LACV infections per year since 1964 (CDC unpublished), though pockets of higher infection risk have been reported [13]. The majority of cases reported occur during the summer months (June, July, and August) [2,29,118]. From 1964 to 1981, there were 1348 cases of California encephalitis (LACV) in the United States of which 88.8 percent came from the upper-midwestern states of Ohio, Wisconsin, Minnesota, Illinois, and Iowa [5], while only 2 percent came from the Appalachian region of the United States (North Carolina, Tennessee, and West Virginia). Recently, LACV infections have been recognized as an emerging disease in the Appalachian region of the United States [6,8,9].

The geographic distribution of the virus is focal [11,13,14,119,120,121], coinciding with the presence of the virus, vector species, and amplification hosts

[2,4,17,20,21,25,27,28,31,81,82,86,114]. The proximity to hardwood forests and their associated tree-holes as well as artificial containers are well-documented risk factors for disease transmission [11,16,22,30,117,122]. It is probable that a large percentage of the reported cases of LACV infections occur in the peridomestic areas, following human encroachment into disease foci [11,14,29]. The recent establishment of two possible accessory vector species, *Ae. albopictus*, and *Ae. japonicus*, into LACV endemic regions could result in an increase the transmission and/or maintenance of the virus within peridomestic areas and/or hardwood forests [6,16,17,19,38,97,123], and could result in an expansion LACV distribution.

LACV was first isolated in 1964 [1], and has become one of most common causes of pediatric arboviral infections in the United States [2,3]. The majority of cases occur in children 15 years and younger [4,5,29,118], with approximately 64 percent of these cases occurring in males [5]. The incidence risk in children 15 years and younger is believed to be 20 to 30 cases per 100,000 persons in endemic areas, with a case fatality rate of less than 1 percent [5,8,118]. However, the true incidence of LACV infections is unknown as cases are typically under diagnosed, under-reported, and some are asymptomatic [2,13,29,36,124].

At present the majority of cases of LACV infections remain undiagnosed [2]. From the results of serosurveys conducted in endemic regions it is clear that the risk of asymptomatic infections are much higher than symptomatic infections, with estimates of asymptomatic infections to clinical infections in pediatric populations ranging from 2:1 to 1500:1 [13,36,124]. The high percentage of asymptomatic infections remains unclear, but results from a recent study [103], provide further evidence that dose of virus and

immune response may responsible for the severity of infection. Previous work on histocompatibility antigens in LACV endemic areas [125], supports this hypothesis. The incidence of LACV infections is therefore much higher, as only the most severe cases typically present for medical care and hence get reported. The results from these studies suggest that there are most likely several hundred thousand infections per year in the United States [13,36,124].

Clinical Overview

Clinical Presentation

LACV is typically responsible for a relatively mild febrile illness in humans, though in a sub-group of patients LACV may result in the development of severe meningoencephalitis [8,43,44,45]. In these patients following the development of febrile illness, there is may be a progressive decline in mental status, with symptoms such as obtundation, grand mal seizures, coma, and death [29,118]. Following the development of coma, recovery has been associated with a variety of neuropsychiatric sequelae, including: neuromotor retardation, developmental retardation, permanent seizures, and personality disorders [8,43,44,45,126,127,128,129,130].

It is likely that the vast majority of patients suffering from LACV infections never present for medical treatment. Those presenting for medical treatment have a febrile illness, associated with myalgias, fever and headache, and are most likely diagnosed as having a viral syndrome [8,41,45,46]. No specific treatment is provided for these patients, and only a small subgroup are hospitalized. Initial evaluations frequently

demonstrate elevation of white blood cell (WBC) count together with headache. These findings raise suspicion of bacterial meningitis, tick-borne diseases, or other nonspecific viral syndromes. Most patients recover within a few days and are released without any specific treatment or need for long-term follow-up.

A small subset of patients develop a progressively severe illness. This is frequently heralded by the development of hyponatremia [8], most likely as a result of the syndrome of inappropriate antidiuretic hormone secretion (SIADH). The decline in serum sodium is associated with a decline in clinical condition, and hyponatremia has been found in 22.6 percent of patients who had a serum sodium of less than 132 mmol/L [8]. Other factors associated with a decline in clinical status include vomiting, seizures at the time of hospitalization, and a temperature of greater than 38.5°C [8,45]. Vomiting has been shown to have a strong correlation with a decline in neurological status and is likely due to the development of encephalitis [8]. Close clinical monitoring and intervention in patients with suspected LAC encephalitis includes serial monitoring on the Glasgow coma scale, with early admission to the ICU for all patients with confusion or disorientation. A Glasgow coma scale score of 8 or less may be an indication for early endotracheal intubation. This subgroup of seriously ill patients need close monitoring in an intensive care setting.

Hyponatremia may be an indication of SIADH and may indicate raised intracranial pressure [8,131], and evidence of increased intracranial pressure and resultant central nervous system herniation may be associated with encephalitis. Mechanical ventilation with intracranial pressure monitoring may be necessary in these patients. No published studies have been performed to evaluate the benefits of intracranial pressure

monitoring, though measures necessary to control elevations of intracranial pressure have been used in the management of this clinical emergency [8,131]. These measures have included hyperventilation using a mechanical ventilator, in combination with drugs to reduce intracranial pressure.

Seizures are a manifestation of severe LACV infections, and are most likely a result of encephalitis associated with increased intracranial pressure. In an early clinical study [43], 51 percent of patients developed seizures, 34 percent suffered grand mal seizures and 17 percent suffered focal seizures. Six patients developed paralysis during encephalitis, all but one resolving by the time of discharge. Of those patients who demonstrated seizures at the onset of illness 33 percent had permanent seizures as a sequela. Most other neurologic symptoms resolved by the time of discharge. In a more recent study reviewing 127 children with severe LACV infections [8], 66 percent had abnormal electroencephalograms (EEG), 46 percent had seizures, and 2 percent had episodes of status epilepticus. Central nervous system herniation was present in 2 percent of patients.

In 2001, a blinded case control study of suspected central nervous system viral infections was performed in eastern Tennessee [16]. Patients in the study received lumbar puncture, and bacterial meningitis was excluded. Serology was performed for all major viral groups both on both sera and cerebrospinal fluid. Diagnostic confirmation of infection was determined by performing paired acute and convalescent sera, and the interpretation of the tests was made using standard guidelines [132]. There were no statistically significant differences in the presence of fever, headache, vomiting, or behavioral changes between the two groups.

Following the earlier case control study [16], a study to compare the clinical symptoms between LACV infection and enteroviral central nervous system (EV-CNS) infections in was carried out in eastern Tennessee [41]. Symptoms in the patients with LACV infection included elevated temperature, headache, photophobia, vomiting, and in severely ill patients, aphasia, seizures, and coma. None of the patients with EV-CNS infections suffered aphasia, loss of consciousness, or seizures. Only one patient with an EV-CNS infection required ICU admission. Of the LACV patients, the following complications were described: aphasia in 33 percent, loss of consciousness in 40 percent, seizures in 40 percent, ICU admission in 27 percent, and requiring mechanical ventilation in 13 percent. LACV were significantly more likely than EV-CNS infections to have aphasia ($p = 0.001$), loss of consciousness ($p = 0.001$), seizure ($p = 0.001$), and admission to a pediatric ICU ($p = 0.05$). Though some clinical similarities exist, LACV infections are associated with significantly higher morbidity than non-polio virus EV-CNS infections.

Laboratory Abnormalities

Many viral infections are associated with reduced white count and absolute lymphocytosis. Patients with LACV infections have experienced WBC counts from peripheral blood ranging from 6.8 K to 49K [8,43,45]. Many of these patients demonstrated a significant number of polymorphonuclear leukocytes, representing 50 percent or more of the total WBC. These findings make it difficult to establish a clear working diagnosis, and are more suggestive of a white count seen in bacterial meningitis.

Cerebrospinal fluid (CSF) counts in the majority of patients presenting with severe LACV infections are abnormal. In one study [43] of 35 patients, 89 percent had white cell counts ranging from 6 to 300; 14 percent had less than 50 percent lymphocytes, and 89 percent had protein levels ranging from 68 to 83 mg/dL. In a later study [45] of 48 patients, the range of white cells in the CSF was 0 to 600 cells, and of these 66 percent were mononuclear, and 31 percent were polymorphonuclear leukocytes. CSF protein levels were elevated in 30 percent of patients, though none demonstrated a low CSF glucose level. In the most recent study involving the largest case-series to date [8], the CSF white cell count ranged from 2 to 867, with lymphocytes predominating in most cases; 25 percent of these cases also demonstrated significant red cells in the CSF. Initial lumbar puncture findings ranged from normal to abnormal. However, by the third day of the illness, significant elevations of white cells in the spinal fluid existed in most patients. These findings illustrate the wide variation in the number and distribution of white cells present in the CSF of patients suffering from LACV infections. These findings also indicate that many samples will contain polymorphonuclear leukocytes, raising early suspicion of early bacterial meningitis.

Diagnosis

Confirmed and probable cases of LACV infections are required to meet both the clinical and laboratory requirements set by the Centers for Disease Controls and Prevention's case definition for neuroinvasive domestic arboviral diseases [132].

The most important factor in establishing a diagnosis of LACV infections is an index of suspicion eg. time spent outdoors, presence of mosquitoes. Additionally,

physicians need to be aware of any clusters of cases consistent with LACV infection. This is particularly important as clusters of cases may occur within a given county or township [11,14].

Hemagglutination-inhibition (HI) tests are the most likely reliable means of establishing a diagnosis in a patient suffering from LACV infection, but only after 5 days [133,134]. Unfortunately, these tests are frequently delayed and hinder making an early diagnosis. The presence of IgM, with the absence of IgG is an indication of acute infection. A fourfold rise in titer drawn 4 to 6 weeks following the initial titer provides confirmation of this diagnosis. High titer IgG tests are an indication of recent infection usually having occurred within the past 3 to 6 months. Complement-fixation (CF) titers are relatively insensitive in establishing early diagnosis of this disease and should never be used alone in establishing the diagnosis of LACV infections [133]. HI, CF, and neutralization plaque reduction assay results showed that neutralization assay was the most sensitive, followed closely by HI reaction [133]. An IgM titer equal to or greater than 1:8 is considered positive in making a presumptive diagnosis. Only neutralization assay and HI reliably showed IgM titers of greater 1:8 within the first 5 days and would provide for a presumptive diagnosis of infection [133].

More recently, enzyme-linked immunosorbent assay (ELISA) using an IgM capture assay, performed by state health departments and the CDC, has been utilized to provide rapid diagnosis. The advantages of these tests are that they provide a high sensitivity and specificity, and can be used to screen large numbers of samples. Currently, the only FDA commercially approved test for LACV is an indirect immunofluorescent assay for IgG and IgM antibodies (Focus Diagnostics, Cypress, CA).

The specificity of this assay is 100 percent, and the sensitivity has been shown to be 83 percent at the time of admission, rising to 93 percent if a second sample was provided within 5 days [118]. This test can be performed rapidly and is effective in making an early diagnosis of LACV infection. The disadvantage of this test is that health departments or reference laboratories typically perform it, as most hospital laboratories will not perform this test due to its associated cost.

CSF viral cultures are an extremely poor means of identifying this virus. Numerous studies demonstrate that the isolation of this virus from spinal fluid is difficult [45], therefore samples of spinal fluid submitted for culture are unlikely to be beneficial in establishing a diagnosis. ELISA assay of CSF using IgM capture has been used in establishing diagnosis [135]. Unfortunately, no commercially available test using PCR to identify LACV infections in humans is available at this time, though recent studies have demonstrated its utility [136,137].

Severe LACV infections have traditionally proved difficult to distinguish from herpes simplex meningoencephalitis and may mimic this disease with similar findings on clinical presentation, CSF analysis, EEG, CT scan, and MRI [29,138,139]. A failure to respond to treatment for herpes simplex encephalitis, or a negative CSF PCR for herpes simplex virus [138], may be an indication to perform additional studies for LACV. In another case results of a brain biopsy performed that showed perivascular mononuclear infiltrates and focal aggregates, suggesting viral encephalitis [8,131]. Symptoms in this case did not suggest herpes simplex encephalitis, though the indirect immunofluorescent monoclonal antibody test to LACV was positive on brain tissue. In an immunocompromised patient thought to have Herpes simplex meningoencephalitis and

who demonstrated negative PCR for Herpes simplex virus, convalescent serology confirmed the diagnosis of a recent LACV infection [139]. Owing to the similarity of the clinical and radiologic findings of encephalitis in patients with Herpes simplex virus and LACV infections during the summer months in endemic areas for LACV, CSF analysis should be submitted for both Herpes simplex virus and LACV. Serologic evaluation for LACV should be simultaneously submitted.

In an early study [128], during acute infection, seizures occurred in 56 percent of hospitalized patients with 13 percent demonstrating persistent seizures. During a one year follow-up of group of 32 patients with confirmed LACV infections and 13 probable infections, 49 percent of patients EEGs were normal, 33 percent were abnormal, and 15 percent indicated abnormalities that were not diagnostic of a seizure disorder. In a more recent study [8], 90 patients underwent EEG with 66 percent were found to be abnormal, focal slowing occurred in 17 percent, lateralization occurred in 9 percent, and focal abnormalities in 3 percent. Those patients with lateralization showed focal abnormalities overlying the temporal lobe. Twenty-eight percent demonstrating results similar to those present in patients with Herpes simplex encephalitis. The presence of these findings, together with radiographic evidence and clinical findings, are consistent with the diagnosis of Herpes simplex encephalitis. This may delay the diagnosis of severe LACV infections.

EEG in patients with LACV often provided evidence of focal changes that may be indistinguishable from those of Herpes simplex encephalitis [8]. These findings, together with abnormalities on CT scan of the brain, or MRI study, may provide confusion in the clinical diagnosis between LACV and Herpes simplex infections. EEG therefore

provides confirmatory evidence of a significant brain lesion, but provides no specificity of diagnosis.

In a recent study [130], a group of children suffering from periodic lateralizing epileptiform discharges (PLEDS) were evaluated. PLEDs patients remain a small subgroup of LACV infection patients, but the results showed that this subgroup of patients had a higher incidence of permanent sequelae including: permanent seizures, developmental issues, and other neurological complications. The need for more aggressive life support was necessary in 88 percent of these patients, while only 21 percent of patients without PLEDs needed such aggressive management. The length of critical care was also prolonged in PLEDs patients.

Treatment

Patients who early in the illness suffer from seizures, altered mental status, vomiting, hyponatremia, and have temperatures of greater than 38.5°C, should be monitored extremely closely, preferably in an ICU. Hyponatremia is usually a manifestation of SIADH and is frequently associated with the early development of encephalitis. This group of patients should be considered medically unstable and monitored in an ICU [8,43,45,139], and these patients may fall into a group who are indistinguishable from acute Herpes simplex meningoencephalitis [138]. Development of focal neurologic signs and intractable seizures may well be associated with a rise in central nervous system pressure. Monitoring with an intracranial-pressure monitoring device may allow for aggressive management of elevated intracranial pressure using osmotic agents and hyperventilation [8,118]. The mainstay of current therapy is

aggressive life support, maintenance of adequate electrolyte balance, early detection and prevention of hyponatremia, mechanical ventilation, and placement of an intracranial-pressure monitoring device when needed [29,118].

Currently, there are no clinically approved medications to treat LACV infections. From the limited body of knowledge it appears that ribavirin may be a treatment option for LACV infections [131,140]. Based on *in vitro* studies where ribavirin was shown to be active [140], the drug was made available as a compassionate release by the FDA for patients suffering from life-threatening LACV infections. Anecdotal evidence suggests that this may have been beneficial in a few patients, but experience is limited [131].

The most likely target of ribavirin in LACV infections is RNA-dependent RNA polymerase, with an *in vitro* minimum inhibitory concentration (MIC) of 0.3 $\mu\text{mol/L}$ [140]. This level is achievable, and high dose oral therapy has obtained levels of 9.5 $\mu\text{mol/L}$ in the CSF [141,142]. In a previous study evaluating the use of ribavirin in HIV infected children, it was demonstrated that doses of 10 mg/kg per day of oral ribavirin resulted in steady state plasma levels of 4 $\mu\text{mol/L}$ [143]. These levels should be therapeutically effective in the treatment of LACV infections.

Timely therapeutic intervention would have the potential benefit of reducing the severity of illness produced by LACV, including its most serious complications. The use of oral ribavirin as a treatment of LAC viral infections may be effective due to its known anti-viral activity against LACV even in low concentrations [144]. Limited experience in treating LAC encephalitis supports this [131,140,145]. Additionally, if it oral ribavirin was found to be an effective treatment for LACV infections it would have an advantage over intravenous ribavirin as it is a commercially available product, thus allowing for

early administration and treatment.

Sequelae

Severe LACV infections can result in a variety of sequelae, including seizures, learning disabilities, cognitive deficits, and behavioral changes [8,45,46]. A recent study [8], found that 36 percent of patients had IQ scores ≤ 79 . An early study in Wisconsin found that there were twice as many patients permanently institutionalized for mental disorders with LACV antibodies, than in the general population [146]. Furthermore, when those patients with physiological cerebral defects were excluded from the analysis, the rate increased to three times as many as compared with the general population. It is clear that a range of debilitating sequelae can result in patients suffering from severe LACV infections, and long-term follow-up of this patient group is mandatory.

Socioeconomic Impacts Associated with Infection

The socioeconomic impacts of LACV infections are traditionally borne by the families and those patients who have suffered from severe LACV infections [4,127], due to its focal nature and that the majority of cases are asymptomatic [2]. A recent study in North Carolina measured the economic and social impacts of severe LACV infections [127]. The projected life-long costs of cases in this study suffering from permanent neurological sequelae ranged from \$48,775 to \$3,090,798, and the loss of 12.90 to 72.37 disability adjusted life years (DALYs). DALYs are defined by the World Health Organization (WHO) as “a time-based measure that combines years of life lost due to premature mortality and years of life lost due to time lived in states of less than full health” [147]. These findings of the high economic costs associated with severe LACV

infection are in agreement with previous studies elsewhere [121]. LACV patients may experience a loss of social interactive skills following the course of illness, resulting in the isolation of these patients from their peers [4], leading to difficulties in the school and home environments.

Prevention

The prevention of LACV infections remains a daunting objective due to its complex eco-epidemiology. Prevention of LACV transmission has been demonstrated best in La Crosse, Wisconsin, where following the detection of the virus, a county-wide public education campaign was initiated, which also included the source reduction and the closure of basal tree-holes to reduce populations of *Ae. triseriatus* [14,148,149,150]. This program greatly reduced the number of reported LACV infections [4,14,148], and in those areas without such preventative efforts there was no demonstrated reduction in the number of reported LACV cases [148]. Vector species and LACV are widely associated with the presence of artificial containers [12,16,17,19,30,34,148,151], though their removal remains challenge without funding and public education efforts. The use of mosquito repellants should be used by those in LACV endemic areas [118]. Ultimately, the avoidance of wooded areas, artificial containers, vector species, and endemic foci, and the use of mosquitoes repellants would reduce the risk of exposure to virus [2,4,16,29,30,116,118,148,152]. Future studies are needed to detect areas of highest risk for virus transmission.

SECTION II: EPIDEMIOLOGY AND CLINICAL FEATURES

CHAPTER III

The Incidence Risk, Clustering, and Clinical Presentation of La Crosse Viral Infections in the eastern United States, 2003-2007

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My contributions to this paper are 1) substantial contribution to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published.

Abstract

Although La Crosse virus (LACV) is one of the most common causes of pediatric arboviral infections in the United States, little has been done to assess its geographic distribution, identify areas of higher risk of disease, and to provide a national picture of its clinical presentation. Therefore, the objective of this study was to investigate the geographic distribution of LACV infections reported in the United States, to identify hot-spots of infection, and to present its clinical picture. Descriptive and cluster analyses were performed on probable and confirmed cases of LACV infections reported to the Centers for Disease Control and Prevention from 2003-2007. A total of 282 patients had reported confirmed LACV infections during the study period. Of these cases the majority (81 percent) presented during the summer, occurred in children 15 years and younger (83.3 percent), and were found in male children (64.9 percent). Clinically, the infections presented as meningoencephalitis (56.3 percent), encephalitis (20.7 percent), meningitis (17.2 percent), or uncomplicated fever (5 percent). Deaths occurred in 1.9 percent of confirmed cases, and in 8.6 percent of patients suffering from encephalitis. The majority of these deaths were in patients 15 years and younger. The county-level incidence risk among counties ($n = 136$) reporting both probable and confirmed cases for children 15 years and younger ($n = 355$) ranged from 0.2 to 228.7 per 100,000 persons. The southern United States experienced a significantly higher ($p < 0.05$) incidence risk during the months of June, July, August, and November than the northern United States. There was significant ($p < 0.05$) clustering of high risk in several geographic regions with three deaths attributed to complications of LAC encephalitis occurring in two of these hot-

spots of infections. Both the incidence risk and case fatality rates were found to be higher than previously reported. We detected clustering in four geographic regions, a shift from the prior geographic distributions, and developed maps identifying high-risk areas. These findings are useful for raising awareness among health care providers regarding areas at a high risk of infections and for guiding targeted multifaceted interventions by public health officials.

Introduction

La Crosse virus (LACV) is a member of the genus *Orthobunyavirus*, family Bunyaviridae, and is the causative agent of LACV infections. LACV was first isolated in 1964 [1], and has become one of most common causes of pediatric arboviral infections in the United States [2,3]. The majority of LACV infections are transmitted to humans through the bite of the primary vector, the eastern tree-hole mosquito, *Aedes triseriatus* [20,25]. The virus is maintained in nature through a vertical and horizontal transmission cycle involving *Ae. triseriatus* [21,22,26] and the primary amplification hosts: the eastern chipmunk, *Tamias striatus*, the gray squirrel, *Sciurus carolinensis*, and the fox squirrel *Sciurus niger* [27,28].

LACV has traditionally been associated with forested areas in the upper-midwestern United States [4], but more recently as an emerging disease in the Appalachian region of the United States [6,8,9]. The majority of cases occur in children 15 years and younger [4,5,29,118], with an average of 79 nationally reported cases per year since 1964, pockets of higher infection risk have been reported [13]. The incidence risk in children 15 years and younger was believed to be 20-30 cases per 100,000, with a

case fatality rate of less than 1 percent [5,8,118]. However, the true incidence of LACV infections are unknown as cases are typically under diagnosed, under-reported, and some are asymptomatic [13,29,36,124]. In this study, we examined probable and confirmed cases of LACV infections from 2003-2007, to determine incidence risk, case fatality rates, and to assess the current spatial patterns of disease risk so as to identify those areas of highest risk for the implementation of future disease control strategies.

Methodology

Study area. Our study area encompassed the eastern United States, the geographic region that includes the majority of previously reported LACV infections and the range of the primary vector, *Ae. triseriatus* [2,5,29,87]. County level incidence risks were calculated and spatial analyses performed on 24 states in the study area. Seventeen of the 24 states reported probable and confirmed cases of LACV infections in children 15 years and younger (Table 1). The following states were part of the study area but did not report any LACV infections in children 15 years and younger during the study period: Arkansas, Delaware, Florida, Maryland, Missouri, New Jersey, and Pennsylvania. All of these states have reported LACV infections in the past.

Disease data. This study was conducted for all states reporting probable and confirmed cases of LACV infections in the United States, 2003-2007, through the ArboNET surveillance system [153]. The ArboNET surveillance system was established by the Centers for Disease Control and Prevention in 2000 to monitor the spread of West Nile virus in the United States. In 2003, the system was expanded to collect data on other arboviral diseases. Through ArboNET, participating health departments report human

cases of arboviral disease. The ArboNET system was queried to search for all LACV infections.

Personal identifiers of patients were deleted before database construction. Clinical and epidemiological LACV data for 151 probable and 275 confirmed cases of LACV infections reported during this time period were provided by the Centers for Disease Control and Prevention. Cases that acquired infection outside of the county/state were excluded from spatial analyses (n = 10).

Case definition. Confirmed cases of LACV infections are required to meet both the clinical and laboratory requirements set by the Centers for Disease Controls and Prevention's case definition for neuroinvasive domestic arboviral diseases [132]. This definition is reprinted below:

In the absence of a more likely clinical explanation as documented by a physician, confirmed cases must meet all of the following criteria:

Clinical Criteria

- 1) Fever, AND
- 2) Acutely altered mental status, or other acute signs of central or peripheral neurologic dysfunction, or pleocytosis associated with illness clinically compatible with meningitis, AND

Laboratory Criteria

- 3) a four-fold or greater change in virus-specific serum

antibody titer, or isolation of virus from or demonstration of specific viral antigen or genomic sequences in tissue, blood, CSF, or other body fluid, or virus-specific immunoglobulin M (IgM) antibodies demonstrated in CSF by antibody-capture enzyme immunoassay (EIA), or virus-specific IgM antibodies demonstrated in serum by antibody-capture EIA and confirmed by demonstration of virus-specific serum immunoglobulin G (IgG) antibodies.

Probable Case Criteria

Cases that met the clinical definition and had stable (less than or equal to a two-fold change) but elevated titer of virus-specific serum antibodies, or virus-specific serum IgM antibodies detected by antibody-capture EIA but with no available results of a confirmatory test for virus-specific serum IgG antibodies in the same or a later specimen, are deemed probable.

Population and geographic data. The 2005, United States Census Bureau, Estimated County Population Dataset [154], was used to calculate the total population and the population 15 years and younger for each county. These populations were used to provide the denominators for calculating incidence risk at the county level. As the US Census Bureau does not provide yearly population estimates that include sex, age, and race

for the county level, the 2000 United States Census [155] was used to provide the denominators for calculating the sex - age - and race - specific incidence risk for all counties reporting LACV infections. Geographic boundary files were downloaded from the United States Census, TIGER, Geodatabase [156], and used for all cartographic displays.

Statistical and geographic analyses. Incidence risk was calculated and spatial analyses were performed on 123 probable and 232 confirmed cases 15 years and younger and on 275 confirmed and 151 probable cases for all ages occurring during the study period for which county level data were available (Table 1). Incidence risk was calculated for all counties in the study area ($n = 1924$) and for counties reporting both confirmed and probable cases of LACV infections under the age of 15 ($n = 136$) and for all ages ($n = 161$). Incidence risks were expressed as the number of cases per 100,000 persons.

To determine if there was a significant difference in incidence risk by month between states reporting cases in the northern ($n = 7$) and southern ($n = 10$) regions of the study area, we calculated the incidence risk by region and month using the both probable and confirmed cases 15 years and younger. The northern region was comprised of Illinois, Iowa, Indiana, Michigan, Minnesota, Ohio, and Wisconsin. The southern region was comprised of Alabama, Georgia, Kentucky, Louisiana, Mississippi, North Carolina, South Carolina, Tennessee, Virginia, and West Virginia.

All incidence risk computations and descriptive analyses were performed using STATA 10.0 [157]. Spatial empirical Bayesian (SEB) smoothing was used to adjust incidence risk due to spatial autocorrelation and high variances resulting from a small number of cases reported in some counties [158,159,160,161]. The resulting smoothed

Table 1. Non-Imported Probable and Confirmed Cases and Incidence Risk*of La Crosse Viral Infections in Children 15 Years and Younger Reported in the eastern United States, 2003-2007, by State

State	Reported Cases	Percentage of Reported Cases	Incidence Risk by State	Range of Incidence Risk Among Counties Reporting Cases
West Virginia	83	23.4	57.8	7.5 - 228.7
Ohio	73	20.6	5.0	0.3 - 37.1
North Carolina	66	18.6	21.0	0.5 - 206.7
Tennessee	50	14.1	18.4	3.6 - 166.6
Wisconsin	21	5.9	12.1	1.1 - 76.7
Illinois	18	5.1	1.3	0.2 - 30.8
Minnesota	9	2.5	10.6	7.3 - 29.8
Virginia	8	2.3	7.8	1.6 - 35.2
Georgia	7	2.0	2.6	0.5 - 129.8
Indiana	6	1.7	2.0	0.5 - 20.6
Louisiana	4	1.1	6.5	4.0 - 23.2
Iowa	3	0.8	41.4	27.7 - 55.1
Kentucky	3	0.8	13.5	7.7 - 23.6
Michigan	1	0.3	1.0	1.0
Mississippi	1	0.3	8.3	8.3
Alabama	1	0.3	0.7	0.7
South Carolina	1	0.3	1.7	1.7
Florida [†]	--	--	--	--
Overall	355	100	7.2	0.3 - 228.7

Table 1. * Incidence risk was calculated in counties reporting probable and confirmed cases of La Crosse viral infections and presented as the number of cases per 100,000 persons in children 15 years and younger, and are expressed here as a range in those states with two or more counties reporting cases.

[†]No incidence risk is reported for states not reporting cases 15 years and younger.

incidence risks allow for better visualization of the spatial patterns compared to the unsmoothed risk.

Global Moran's I [162] and the Moran Local Indicators of Spatial Association (LISA) were used to assess for evidence of spatial clustering [163]. Statistical significance of both global and local Moran's I statistics were tested using 9999 permutations. All spatial analyses were performed using GeoDa Version 0.95i [164], and cartographic displays were done using ArcView GIS 9.2 [165].

Results

Descriptive analyses of cases. A total of 282 patients had confirmed LACV infections reported to the CDC (Table 2). Most cases presented during July (24.9 percent), August (32.6 percent), and September (23 percent). Cases ranged in age from 0.5 to 86, with a median age of 9; 83.3 percent were under the age of 15, and the majority were males (64.9 percent).

The sex-specific incidence risk (per 100,000 persons) for all counties reporting confirmed cases was 1.9 and 1.0 for males and females, respectively. The age-specific incidence risk was 1.2 for children under one year; 5.4 for 1-5 years; 6.8 for 6-10 years; 4.7 for 11-15 years; 0.5 for 16-20 years; and 0.2 for 21 years and older. Blacks had the lowest race-specific incidence risk (0.3 per 100,000 persons) and American Indians had the highest (4.9 per 100,000 persons). The incidence risk for all counties reporting probable and confirmed cases combined in children 15 years and younger was 7.2 per 100,000 persons (mean 30.2), and 1.6 per 100,000 persons (mean 5.8) for the total population.

Table 2. Epidemiological and Clinical Characteristics of Confirmed and Probable La Crosse Viral Infections Reported in the eastern United States, 2003-2007

Variable	Total Confirmed (%)	Probable and Confirmed Cases Combined (%)
Sex		
Male	183 (64.9)	264 (60.6)
Female	99 (35.1)	172 (39.4)
Unknown	--	1
Age		
0.1 – 0.9 yr	3 (1.06)	5 (1.15)
1 yr	11 (3.9)	13 (2.98)
2 – 5 yr	59 (20.9)	98 (22.8)
6 – 10 yr	98 (34.8)	157 (26.4)
11 – 15 yr	64 (22.7)	87 (19.95)
16 – 20 yr	7 (2.5)	15 (3.44)
≥ 21 yr	40 (14.2)	61 (13.99)
Unknown	--	1
Race		
White	241 (95.3)	368 (95.6)
Black or African American	9 (3.6)	11 (2.86)
American Indian or Alaska Native	3 (1.2)	4 (1.04)
Asian	0 (0)	1 (0.259)
Other	0 (0)	1 (0.259)
Unknown	29	52

Table 2 Continued. Epidemiological and Clinical Characteristics of Confirmed and Probable La Crosse Viral Infections Reported in the eastern United States, 2003-2007.

Month of presentation		
March	1 (0.35)	2 (0.458)
April	3 (1.06)	3 (0.686)
May	1 (0.35)	2 (0.454)
June	23 (8.2)	29 (6.64)
July	71 (24.9)	110 (25.2)
August	92 (32.6)	138 (31.6)
September	65 (23)	105 (24.0)
October	23 (8.2)	44 (10.1)
November	2 (0.71)	3 (0.686)
December	1 (0.35)	1 (0.229)
Clinical manifestation		
Meningioencephalitis	157 (56.3)	242 (55.3)
Encephalitis	58 (20.7)	78 (17.8)
Meningitis	48 (17.2)	87 (19.9)
Uncomplicated fever	14 (5.0)	18 (4.4)
Other	2 (0.7)	3 (0.7)
Unknown	3	8
Death	5 (1.86)	6 (1.43)
Unknown*	13	16

Table 2. *Unknown, represents the number of confirmed and probable cases for which the case outcome was not reported.

The incidence risk was significantly higher in the states in the southern region than those states in the northern region for June ($p = 0.0018$), July ($p = 0.0002$), August ($p = 0.0033$), and October ($p = 0.0053$). There were no statistically significant difference ($p > 0.05$) in the incidence risk between the states in the northern and southern regions for the months of March, April, May, September, and November.

Clinically, the infections presented as meningioencephalitis (56.3 percent), encephalitis (20.7 percent), meningitis (17.2 percent), uncomplicated fever (5 percent), or other (0.7 percent) (Table 2). Deaths accounted for 1.9 percent of confirmed cases, and were reported in Indiana, Michigan, Tennessee, and West Virginia. All deaths occurred in patients suffering from LAC encephalitis (100 percent). Of those patients presenting encephalitis 8.6 percent died. The majority (80 percent) of these deaths were in patients 15 years and younger (median age 6, range 4-86) and occurred in males (80 percent). Three deaths occurred in the high risk clusters in West Virginia and in Tennessee.

Spatial distribution. In children 15 years and younger (probable and confirmed combined), state-level incidence risk ranged from 0.7 to 56.4 per 100,000 persons whereas at the county-level incidence risk in counties reporting cases, ranged from 0.2 to 228.7 per 100,000 persons (Table 1). Geographically, the highest incidence risks were observed in western and central Illinois, northeastern Iowa, south central Minnesota, southwestern Wisconsin, and the Appalachian region (south central Kentucky, south central Ohio, western North Carolina, central and eastern Tennessee, south central West Virginia) (Fig 1). The spatial patterns are more easily recognizable when smoothed (Fig. 1b) as compared to the unsmoothed incidence risk map (Fig. 1a). The global Morans I value for children 15 years and younger, was 0.1904 ($p = 0.0001$) for only confirmed

cases and 0.2223 ($p = 0.0001$) for both probable and confirmed cases combined. Forty-seven of the counties in the study area showed evidence of significant high incidence risk ($p < 0.05$) detected by LISA using confirmed cases, while 54 counties had evidence of significant clustering of high risk ($p < 0.05$) detected using both probable and confirmed cases. The spatial patterns for both confirmed cases and for probable and confirmed cases combined were similar. Therefore, only the spatial patterns using both the probable and confirmed cases are presented in Figure 2. The clusters detected by using both probable and confirmed cases combined were found in northeastern Iowa/southwestern Wisconsin, and in south central Ohio, western North Carolina/eastern Tennessee/northeastern Georgia, and south central West Virginia/eastern Kentucky/northwestern Virginia (Fig 2). County-level incidence risks for counties that were part of the significant high clusters ranged from 4.7 to 228.7 per 100,000 persons (mean 54.6) in children 15 years and younger.

Discussion

Our study provides the first risk map of LACV infections for the United States, and presents insights into the clinical picture of LACV infections. We found both a higher incidence risk than previously reported ranging up to 228.7 cases per 100,000 persons in children 15 years and younger and a case fatality rate of 1.9% [5,7,8]. This study highlights the differences of the clinical presentation of LACV infections as meningioencephalitis, encephalitis, meningitis, uncomplicated fever, or other; rather than the traditional method of reporting of infections as purely LACV encephalitis.

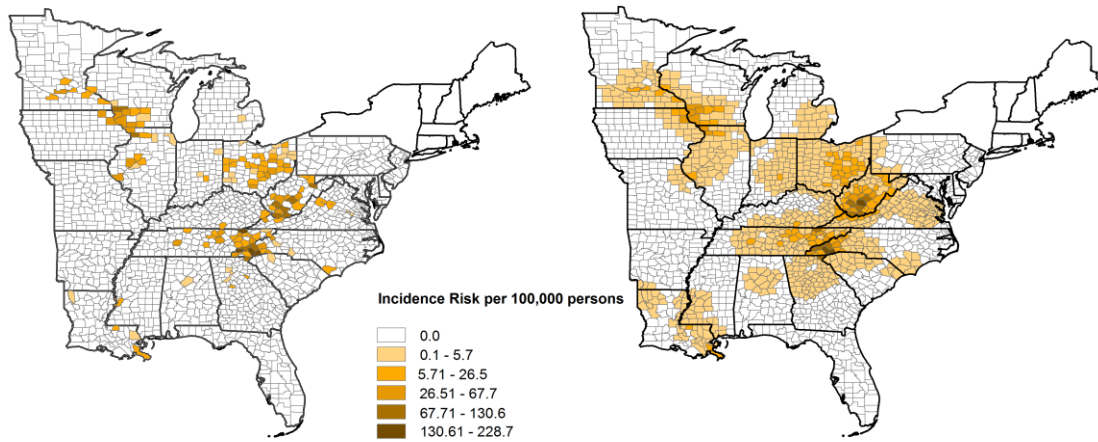


Figure 1. Distribution of unsmoothed and smoothed incidence risk in children 15 years and younger.

The map on the left represents the distribution of a) unsmoothed risk and b) smoothed risk of La Crosse infections at the county level for the eastern United States.

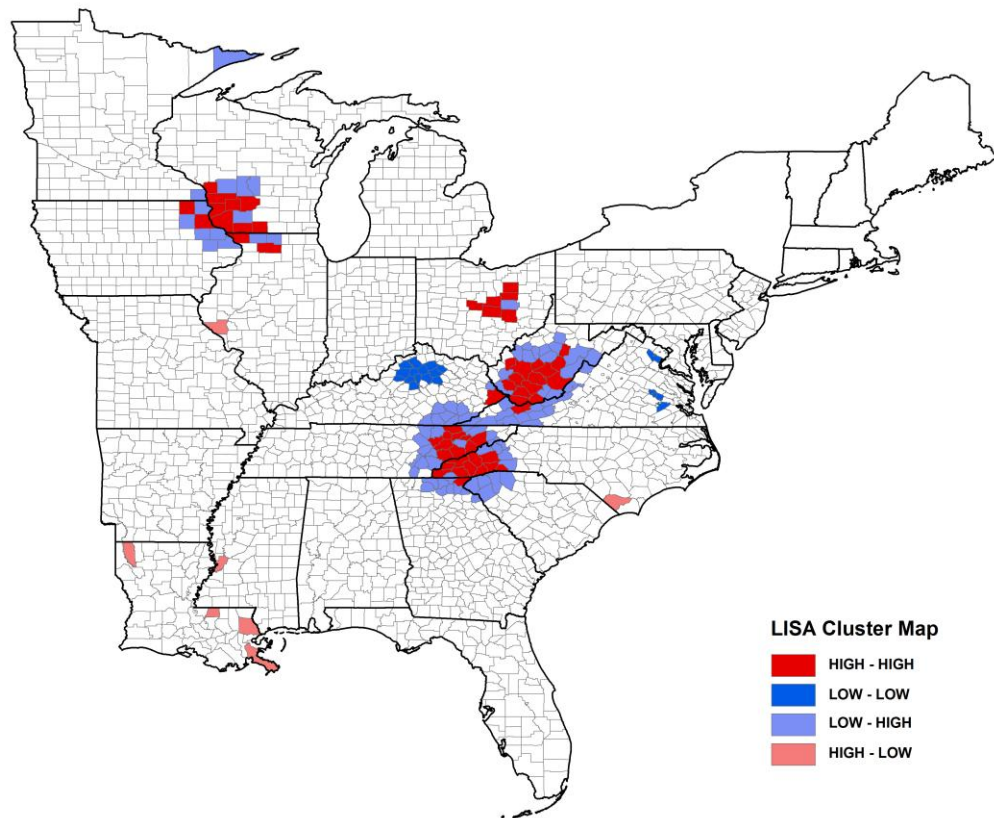


Figure 2. Spatial patterns of disease risk in children 15 years and younger.

This map shows the significant clustering of La Crosse infections at the county level detected by the Moran's I Local Indicators of Spatial Autocorrelation (LISA) for the eastern United States. Four types of spatial autocorrelation are observed using the LISA statistic (High-High, Low-Low, High-Low, and Low-High). Positive spatial autocorrelation is represented by High-High and Low-Low, and negative spatial correlation by High-Low and Low-High. Positive spatial autocorrelation (i.e. an association of areas of similar values) were represented as either High-High (i.e. a high risk in an area surrounded by similarly high values in neighboring areas) or Low-Low (i.e. a low risk in an area surrounded by similarly low values in neighboring areas). Negative spatial autocorrelation (i.e. an association of areas of dissimilar values) was represented as either High-Low (i.e. a high rate in an area surrounded by low values in neighboring areas) or Low-High (i.e. a low rate in an area surrounded by high values in neighboring areas).

To our knowledge this is the first use of smoothing techniques, the global Moran's I, and the Moran Local Indicators of Spatial Association (LISA) to detect spatial clustering of LACV infections at a national level in the United States. We identified high risk clusters in four regions of the United States. These high risk clusters should be targeted for future studies and intervention efforts.

The majority of cases reported occurred in the summer months (June, July, and August) in agreement with previous studies [2,29,118], with a significantly higher incidence risk occurring in southern states during the months of June, July, August, and September. This is likely due to the transmission cycle of LACV, which involves both horizontal and vertical transmission. The burden of infected mosquitoes would continue to increase into the summer months as each successive generation of mosquitoes fed upon infective amplification hosts, followed by increased transovarial transmission of the virus to their progeny. The highest risk of infection would thus occur during the height of this amplification process when the highest burden of infected mosquitoes would be reached. The height of this viral amplification coincides with the summer months when humans are most apt to spend the most time outdoors, thus putting themselves at an increased risk for coming into contact with infected mosquitoes.

Temperature may play a role in higher incidence risk in southern region of the United States. Given that transovarial transmission following one gonotrophic cycle is likely rare in nature [21], orally infected *Ae. triseriatus*, likely need to complete at least two gonotrophic cycles to transovarially transmit the LACV to their eggs [166]. As only a small percentage of *Ae. triseriatus* may survive to complete their second gonotrophic cycle in nature [166], an increase in the ambient temperature would increase the number

of gonotrophic cycles possible in lifespan of a vector. Additionally, higher temperatures would have the added effect of increasing viral dissemination, titers, and transmission in vectors [167,168]. Warmer temperatures, which are traditionally present for longer periods of time in the southern United States could play a substantial role in increasing overall vectorial capacity.

Previous studies have shown that over 90% of reported cases occurred in children 15 years and younger [5], with approximately 64% of these cases occurring in males [5]. Our study confirms these findings with roughly 83 percent of confirmed cases occurring in children 15 years and younger and 64.9 percent of these cases occurring in males. Clinical studies have demonstrated similar findings [8,43,45]. The tendency for children to develop severe LACV infections may be due to a variety of factors including differences in the pediatric and adult immune system, and/or the longer time children may spend outdoors, though such risk factors are not well understood. It has been hypothesized elsewhere that the higher incidence risk in males may be due to the fact that boys tend to spend more time outdoors than girls increasing their risk of contact with infective vectors [116,124].

Of the 426 reported probable and confirmed LACV infections during our study period, the Appalachian region including West Virginia, North Carolina, Ohio, and Tennessee reported 22.2 percent, 20.8 percent, 18.5 percent and 22.2 percent of reported cases, respectively, and accounted for 74.5 percent of all cases during the study period. A previous study from 1964-1981, reported 1348 cases in the United States of which 88.8 percent came from Ohio, Wisconsin, Minnesota, Illinois, and Iowa [5], while only 2% came from North Carolina, Tennessee, and West Virginia. Though our results

encompass only a five-year period, the higher incidence risk in these states may indicate a shift in LAC infections from the upper Midwest to the Appalachian region of the United States [6,8,9]. The reason for this shift is unclear, but could be due to a number of factors including changes in diagnosis, reporting, prevention strategies, as well as changes in the epidemiology of the disease.

Though our finding of an incidence risk of 30.2 per 100,000 persons in children 15 years and younger in probable and confirmed cases combined was similar to previous reports of an incidence risk of 20 to 30 per 100,000 persons in the same age group [10,15]. But our finding of a mean incidence risk in the significantly high spatial clusters was numerically higher, 54.6 per 100,000 persons, then the incidence risk previously reported for the same age group [10,15]. In the individual counties reporting cases of LACV infections we found the incidence risk in children 15 years and younger ranging from 0.16 to 165.4 per 100,000 persons using only confirmed cases, and from 0.2 to 228.7 per 100,000 persons using both confirmed and probable cases combined. These results indicate the wide variation that occurs due to the focal nature of the virus within counties reporting LACV infections, and highlights the need to report the range of incidence risk, rather than reporting only the mean incidence risk which may not provide an accurate assessment of risk within these focal areas. From the results of serosurveys conducted in endemic regions it is clear that the risk of asymptomatic infections is much higher than symptomatic infections, with estimates of asymptomatic infections to clinical infections in pediatric populations ranging from 2:1 to 1500:1 [13,36,124]. The incidence of LACV infections are therefore much higher, as only the most severe cases typically present for medical care and hence get reported. The results from these studies

suggest that there are most likely several hundred thousand infections per year in the United States [13,36,124], but our findings suggest that there are many more cases than previously estimated. The spatial distribution (Fig 1) reaffirms the focal nature of these infections, and identifies the areas with the highest risk.

The use of probable and confirmed cases combined increased overall cluster detection from 47 to 54 counties. The significant high risk clusters detected should be targeted for future studies and for interventions by public health officials.

Reporting the mean incidence risk at the state or county level may lead to a distorted picture of the spatial patterns of LACV infections and thus decrease the perception of risk. Future reporting should include the range of incidence risk occurring in those counties reporting cases. This will minimize misperceptions of risk, as the use of incidence risk continues to remain the most used tool to identify high risk areas for education, prevention, and intervention.

Though LACV infections are typically reported as LAC encephalitis there appears to be four distinct clinical syndromes, as well as asymptomatic infections [2,8,42,43,45], These include the most severe LAC encephalitis, LAC meningioencephalitis, LAC meningitis, and LAC fever. It is difficult to assess the true incidence of these syndromes since they have often been collectively reported as LAC encephalitis in previous reports/studies [43,44,45]. Accurate reporting of the patients' clinical syndrome is necessary to determine and monitor the future rates of disease presentation.

Our study found a case fatality rate of 1.9% in confirmed cases, with all of the deaths occurring in patients presenting encephalitis. This finding is much higher than the case fatality rate of 0.3% reported in a previous study of LACV infections in the United

States [5]. The reason for the higher case fatality rate is unclear, but may be due to a variation in virulence in the LACV strain(s) circulating in this region of the United States [67]. The majority of these were in children 15 years and younger of whom had encephalitis. These findings suggest that LACV infections might be more virulent than sometimes reported in medical literature. Three deaths (probable and confirmed cases combined) occurred in two of the four highly significant clusters of probable and confirmed cases of LACV infections in West Virginia and Tennessee.

Our study has some limitations. Probable and confirmed cases were reported through a passive surveillance system, which inherently suffers under-reporting. Notwithstanding this limitation we feel that the majority of cases progressing to severe illness were diagnosed and reported to public health officials. Using only confirmed cases as well as probable and confirmed cases combined, we were able to demonstrate similar high incidence risk and case fatality rates. We were also able to show similar patterns in disease clustering. Clinical data was reported from multiple state health departments to the CDC, which did not allow for the verification of laboratory results and the diagnosis of the specific clinical presentation for each patient. We feel that this is a small limitation and that the majority of cases reported have been correctly separated into the four manifestations of severe LACV infections by clinicians.

One drawback to using the LISA is the issue of multiple comparisons which increases type I error rates. We didn't make an attempt to adjust for this because some authors have suggested that any adjustments made to reduce the type I errors would increase type II errors [159,169], in turn reducing the test's power to detect truly significant clusters.

Our findings of a incidence risk within significantly high spatial clusters and case-fatality rate indicate a much higher burden of disease than previously reported, and demonstrate that LACV infections are much most common than previously reported. We have demonstrated the usefulness of these spatial statistical techniques to detect hot-spots of infections, thus allowing for targeted interventions by public health officials while raising awareness among health care providers of geographic areas at the highest risk of disease.

Our results will allow focused national serological studies, form the basis for the development of predictive models of virus transmission, provide a methodology for the use spatial analyses at a national level for other infectious diseases, and demonstrate the need for the reporting of arboviral and other disease cases at smaller geographic scales.

CHAPTER IV

Assessing Risk in Focal Arboviral Infections: Are We Missing the Big or Little Picture?

This chapter is a lightly revised version of a paper by the same name *IN REVIEW* at the journal *Public Library of Science One* by Andrew D. Haddow, Carl J. Jones, and Agricola Odoi:

Haddow, A.D., C.J. Jones, A. Odoi. Assessing Risk in Focal Arboviral Infections: Are We Missing the Big or Little Picture? *PLOS One*: In Review

My contributions to this paper are 1) substantial contribution to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published.

Abstract

Focal arboviral infections affecting a sub-set of the overall population present an often over-looked set of challenges in the assessment and reporting of risk and the detection of spatial patterns. Our objective was to assess the possible variation of risk when using different at-risk populations and geographic scales for the calculation of incidence risk and the detection of disease clusters. We explored these variations using a pediatric arbovirus, La Crosse virus, as our model. Descriptive and cluster analyses were performed on probable and confirmed cases of La Crosse infections reported to the Tennessee Department of Health from 1997-2006, using three different at-risk populations and two geographic levels to assess the variation in incidence risk and to investigate evidence of clustering using both global and local spatial statistics. We determined that the most appropriate at risk population to calculate incidence risk, and to assess evidence of clustering was 0-15 year old population cohort. Based on our findings, the most appropriate geographical level to conduct spatial analyses and report incidence risk was the census tract level. Our results indicate the possibility of missing disease clusters resulting from performing incidence risk investigations of focal diseases using inappropriate at-risk populations and/or large geographic scales. Public Health efforts to improve both disease surveillance and health planning would be better improved through the assessment of risk in well defined at-risk populations and geographic scales. This ensures that public health efforts to control disease occurrence are as efficient as possible.

Introduction

The first step in the control and prevention of pathogen transmission requires the identification of the population at-risk. Early work involved the use of purely observational data to identify and prevent disease outbreaks, such as John Snow's calculation of the risk of death by water supply, leading to the identification of "contaminated" water supplies and efforts by the City of London to prevent the drinking of water from those sources [170]. Though today we have more advanced technologies at our disposal, the underlying principles of determining disease occurrence remain roughly the same. We use the incidence of disease to determine populations at-risk and cluster analyses to determine the hot-spots of infections within those populations in an effort to interrupt and/or prevent transmission.

In this study, we explored the variability of incidence risk and investigated evidence of clustering, using a focal arbovirus, La Crosse virus (LACV) as our model. LACV is a member of the genus *Orthobunyavirus*, family *Bunyaviridae*. It is the causative agent of LACV infections, and is the most common cause of pediatric arboviral encephalitis in the United States [2,3], with the majority of cases being reported in children 15 years and younger [4,5,29,118]. Maintenance and transmission of the virus typically occurs in focal wooded areas where the primary vector, the eastern tree-hole mosquito, *Aede triseriatus* [20,21,22,26], and the primary amplification hosts the eastern chipmunk (*Tamias striatus*), the gray squirrel (*Sciurus carolinensis*), and the fox squirrel (*Sciurus niger*), are in close contact [2,27,28]. Transmission to humans occurs when children enter these focal areas and are bitten by infective mosquitoes.

LACV has traditionally been associated with the upper-midwestern United States [4] though it has been reported in other regions; recently as an emerging disease in eastern Tennessee [6,7,16]. There is an average of at least 100 reported cases per year [13]. However, the true incidence of LACV infections are unknown as the disease is under-diagnosed and under-reported [6], making detection and intervention by public health officials problematic.

From 1964-1996, there were 2370 total cases of LACV infections reported to the CDC, with Tennessee reporting 9 cases or 0.38 percent of the total cases. In 1997, a cluster of 10 cases was detected in eastern Tennessee [6], during which time active surveillance for the virus was initiated by both the University of Tennessee and The Tennessee Department of Health. During the ten year time period following increased surveillance efforts, 1997-2006, Tennessee reported 118 cases, out of the 1069 cases reported to the CDC, accounting for 11 percent of all nationally reported cases, marking a significant increase in cases for Tennessee from the previous 32-year time period.

Focal diseases affecting a sub-set of the overall population present an often overlooked set of challenges in calculating and reporting incidence and the detection of spatial patterns. We explored these unique challenges by examining cases of a focal pediatric arbovirus, LACV, in eastern Tennessee to determine the most appropriate at risk population and geographic scale to use in the investigation and reporting of disease risk. It is hoped that the findings of this study will provide information to public health officials and researchers an improved definition of at-risk populations and hence better quantification of disease risk. This information will be useful in guiding health planning decisions to control disease.

Methodology

Study area and disease data. This study was conducted in eastern Tennessee (Figure 3), a region endemic for LACV [6,16]. Case data for probable and confirmed cases of LACV infections from 1997-2006 were provided by the East Tennessee Regional Health Department, upon written release by patients infected with LACV. Variables used in this study were: patient residence, patient age, and infection onset date.

Personal identifiers of patients were deleted before the database was released to investigators. Location data was available for 15 probable and 76 confirmed cases of LACV infections reported during this time period. Probable and confirmed cases (n = 91) were combined for all analyses.

Confirmed cases of LACV infections met both the clinical and laboratory requirements set by the Centers for Disease Controls and Prevention's case definition for neuroinvasive domestic arboviral diseases [132,153]. Cases that met the clinical definition and the initial antibody screening that detected virus specific antibodies, were classified as probable cases.

Population and geographic data. The majority of LACV infections are pediatric [4,5,29,118], therefore it is not appropriate to use the total population as the denominator when calculating risk. The use of 0-18 and 0-15 year old population cohorts would be more appropriate for the calculation of risk for these infections. Thus, the 2000 United States Census was used to calculate the total population, the 0-18 and the 0-15 year old cohorts for each county and census tract. These populations were used to provide the denominators for the calculation of incidence risk at both the county and census tract levels to assess the variations that occur as a result of using different at-risk

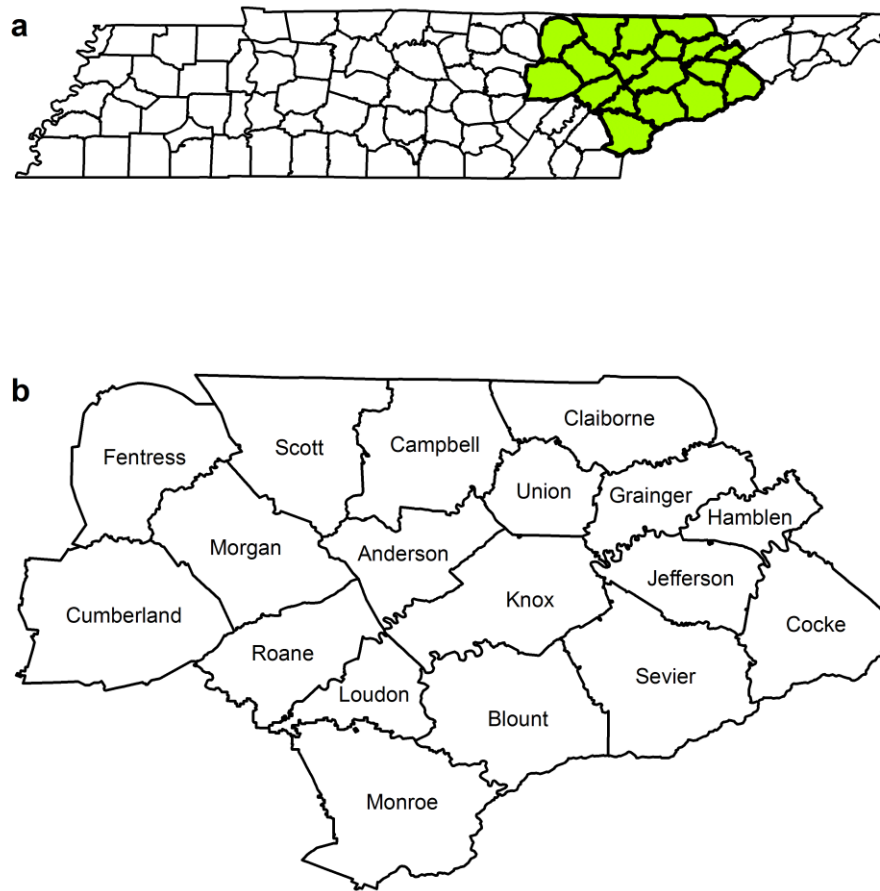


Figure 3. Maps of the study area.

Map of Tennessee (a) showing the study area and (b) the distribution and names of the counties within the study area.

populations.

Geographic boundary files were downloaded from the United States Census, TIGER, Geodatabase [156], and used for all cartographic displays.

Statistical and geographic analyses. The incidence risks were calculated at both the county and the census tract spatial scales and spatial analyses performed on 91 cases (100 percent) of all ages, 86 cases (94.5 percent) that were 0-18 years old, and 84 cases (92.3 percent) that were 0-15 years old, for which location data were available. The incidence risks were calculated for all counties in the study area ($n = 18$), and for counties reporting cases of LACV infections for all three age groups ($n = 14$). Incidence risks were also computed for all census tracts in the study area ($n = 230$), only those reporting cases ($n = 55$), census tracts reporting cases 0-18 years old ($n = 52$), and census tracts reporting cases 0-15 years old ($n = 51$). Incidence risk was expressed as the number of cases per 100,000 persons.

Descriptive analyses and the calculation of incidence risk were performed using STATA 10.0 [157]. To adjust for the high variances resulting from the small number of cases reported in some census tracts we smoothed the risk in the study area using spatial empirical Bayesian (SEB) smoothing [158,159,160,161], implemented in GeoDa [164], using inverse distance spatial weights. This method allowed for the better visualization of spatial patterns compared to the maps of unsmoothed incidence risks at the census tract level.

Evidence of spatial clustering was assessed by the Global Moran's I [162] and the Moran Local Indicators of Spatial Association (LISA) [163], implemented in GeoDa Version 0.95i [164], using inverse distance spatial weights. Statistical significance was

tested using 9999 permutations. Cartographic displays were made using ArcView GIS 9.2 [165].

Results

Incidence risk. Incidence risk varied by the geographic scales and population cohort (Table 3, 4). Higher incidence risks were most evident at the census tract level and in the younger age cohorts (15 years and younger). The incidence risk in the whole population, among counties reporting cases ranged from 0.94 to 46.88 per 100,000 persons (median 8.77), whereas it was 11.86 to 133.33 per 100,000 persons (median 27.72) among census tracts reporting cases (Table 3, 4). In children 0-18 years old, the county-level incidence risk in those counties reporting cases, ranged from 3.91 to 188.37 per 100,000 persons (median 34.93), while it was 44.27 to 547.05 per 100,000 persons (median 114.64) among census tracts reporting cases (Table 3, 4). Among children 0-15 years old, the county-level incidence risk in counties reporting cases ranged from 4.67 to 226.35 per 100,000 persons (median 41.49), whereas the incidence risk in those census tracts reporting cases ranged from 50.89 to 673.85 per 100,000 persons (median 126.74) (Table 4, 5).

Geographically, the highest risks were observed in the western and northeastern counties (Figure 4, 5). However, assessment of the spatial patterns at the census tract level using SEB smoothed rates indicates that within the high risk counties presented in Figure 4, only a few census tracts had a high risk of LACV infections (Figure 5). Thus, most of the census tracts in these seemingly high risk counties actually have a low risk of disease. Based on the smoothed rates it is obvious that the geographic areas of highest

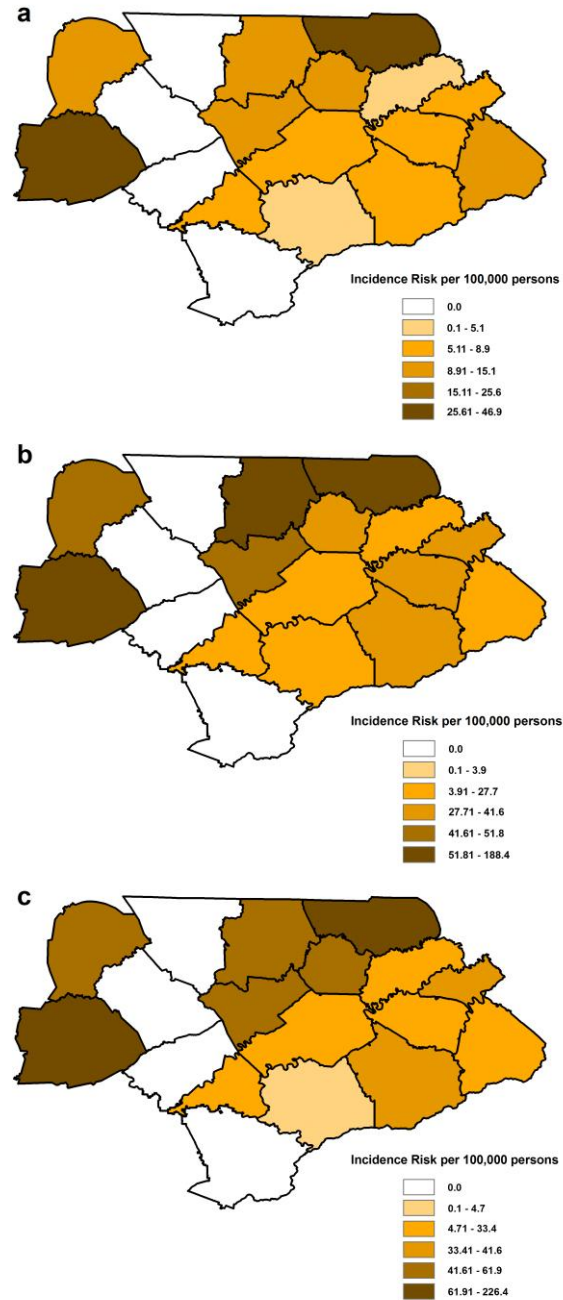


Figure 4. The unsmoothed incidence risk at the county level.

These maps represent the distribution of unsmoothed risk of La Crosse infections at the county level for eastern Tennessee using three different populations to calculate incidence risk: a) total population, b) population 18 and younger, c) population 15 and younger.

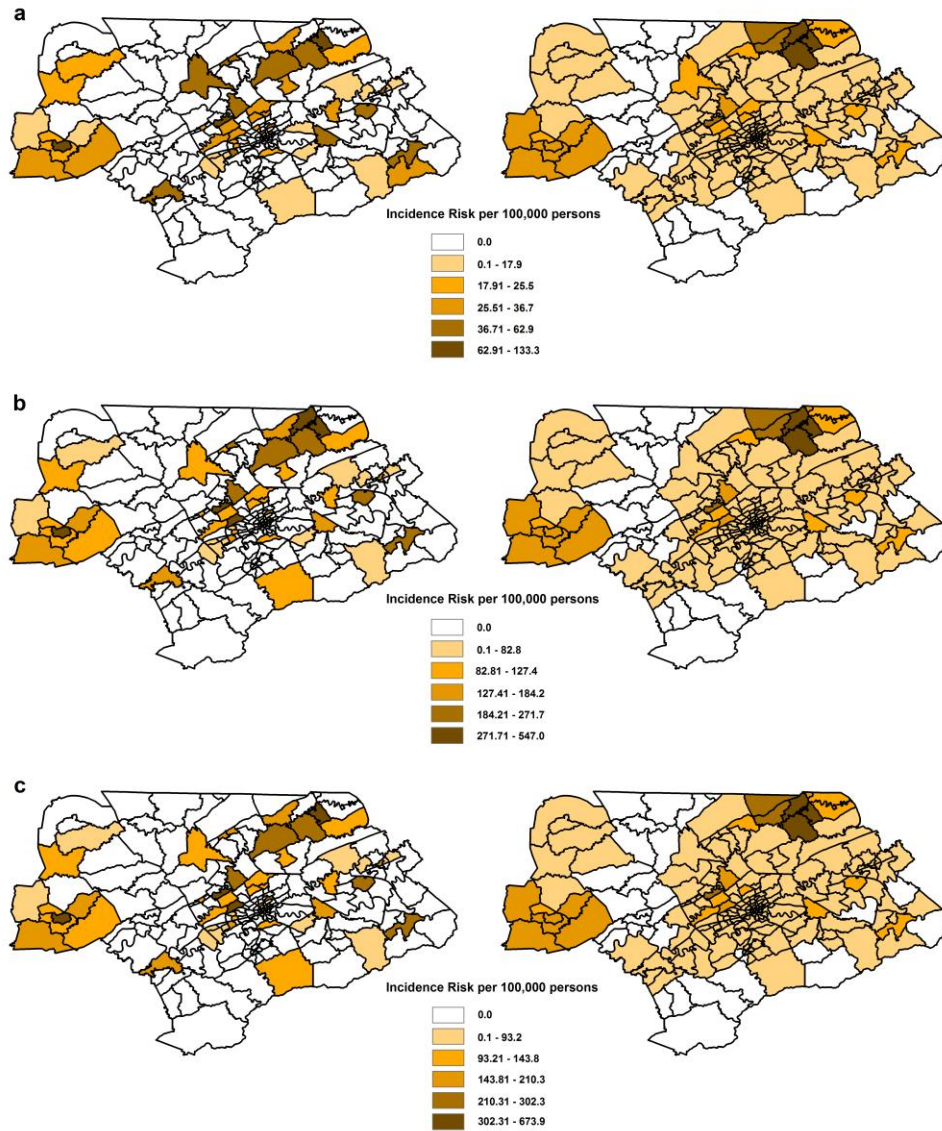


Figure 5. The unsmoothed and smoothed incidence risk at the census tract level.

The maps in the left hand column represent the distribution of unsmoothed risk of La Crosse infections at the census tract level for eastern Tennessee. The maps in the right hand column represent the distribution of spatial empirical Bayesian smoothed risk for La Crosse infections in the eastern Tennessee at the census tract level. Incidence risk was calculated using three different population sets: a) total population, b) population 18 years and younger, c) population 15 years and younger.

Table 3. Comparisons of the Incidence Risk of La Crosse Infections for Three at Risk Populations at the County Level in eastern Tennessee, 1996-2006

At Risk Population	Obs.	Incidence Risk per 100,000 persons			
		Median	Range	Mean	Variance
Total population (All Counties)	18	7.60	0 – 46.88	9.65	129.47
Total population (Only Those Counties Reporting Cases)	14	8.77	0.94 – 46.88	12.40	132.50
0-18 year old population cohort (All counties)	18	26.26	0 – 188.38	38.17	2166.79
0-18 year old population cohort (Only Those Counties Reporting Cases)	14	34.93	3.91 – 188.37	49.07	2257.14
0-15 year old population cohort (All counties)	18	31.43	0 – 226.35	44.79	3097.14
0-15 year old population cohort (Only Those Counties Reporting Cases)	14	41.49	4.67 – 226.35	57.59	3256.33
Obs. = Observations, and refer to the number of counties for the specific analysis					

Table 4. Table Comparisons of the Incidence Risk of La Crosse Infections for Three at Risk Populations at the Census Tract Level in eastern Tennessee, 1997-2006

At Risk Population	Obs.	Incidence Risk per 100,000 persons			
		Median	Range	Mean	Variance
Total population (All Census Tracts)	230	0	0 – 133.33	8.13	339.59
Total population (Only Those Census Tracts Reporting Cases)	55	27.72	11.86 – 133.33	33.99	544.87
0-18 year old population cohort (All Census Tracts)	230	0	0 – 547.05	33.82	6242.02
0-18 year old population cohort (Only Those Census Tracts Reporting Cases)	52	114.64	44.27 – 547.046	149.57	10374.16
0-15 year old population cohort (All Census Tracts)	230	0	0 – 673.85	37.45	8088.70
0-15 year old population cohort (Only Those Census Tracts Reporting Cases)	51	126.74	50.89 – 673.85	168.90	14401.27

Obs. = Observations, and refer to the number of census tracts for the specific analysis

risk are clustered in a few census tracts in the western and northeastern parts of the study area. This pattern seems to persist across all three age groups studied.

Geographic hot-spots of infection. Spatial analyses using the Global Morans I and the LISA were first performed at the county level, using all three at-risk population cohorts. No significant ($p > 0.05$) spatial clustering was demonstrated using the global statistic for the county level analysis using all at-risk population cohorts. However, the LISA analysis revealed, significant clusters ($p < 0.05$) using both the total at risk population and 0-18 year old cohort, with each reporting the same cluster in Union County. No significant high risk clusters ($p > 0.05$) were detected using the 0-15 year old population cohort. At the census tract level, the global Morans I value was 0.1753 ($p = 0.0004$), 0.1801 ($p = 0.0008$), and 0.1863 ($p = 0.0007$) using the total at risk population, 0-18 and 0-15 year old age cohorts, respectively. Twelve census tracts in the study area displayed evidence of significant clustering of high incidence risk ($p < 0.05$) detected by LISA, for all the three at risk population cohorts (Figure 6). Four types of spatial autocorrelation are observed using the LISA statistic (High-High, Low-Low, High-Low, and Low-High). Positive spatial autocorrelation are represented by High-High and Low-Low, and negative spatial correlation by High-Low and Low-High. Positive spatial autocorrelation (i.e. an association of areas of similar values) were represented as either High-High (i.e. a high risk in an area surrounded by similarly high values in neighboring areas) or Low-Low (i.e. a low risk in an area surrounded by similarly low values in neighboring areas). Negative spatial autocorrelation (i.e. an association of areas of dissimilar values) was represented as either High-Low (i.e. a high rate in an area surrounded by low values in neighboring areas) or Low-High (i.e. a low rate in an area

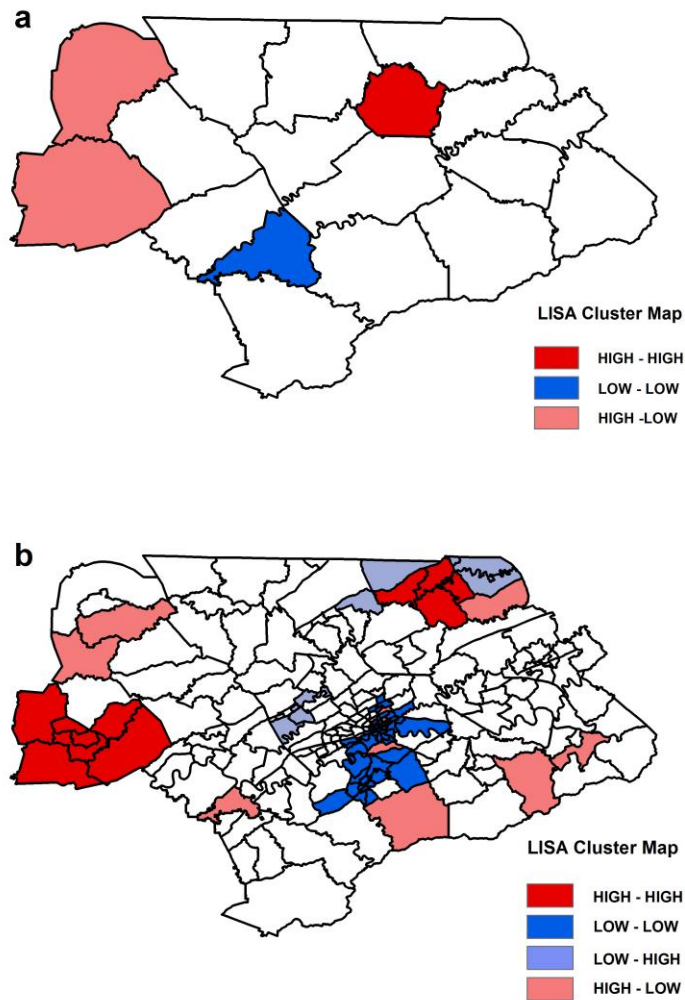


Figure 6. The spatial clustering of La Crosse virus infections at the county and census tract levels.

These maps show the significant clustering of La Crosse infections at the county level for the total population and the 0-18 year old cohort (a) and at the census tract level for all three population cohorts (b) detected by the Moran's I Local Indicators of Spatial Autocorrelation for eastern Tennessee.

surrounded by high values in neighboring areas). Significant high risk clusters were detected in Claiborne County (4 census tracts) and in Cumberland County (8 census tracts). The incidence risk in the 12 census tracts displaying evidence of significant high risk spatial clustering ranged from 67.98 to 673.85 (median 177.62) per 100,000 persons for the 0-15 year old population cohort, with the other at-risk populations displaying a similar high incidence risk (Table 5).

Discussion

In this paper, we have presented evidence of the variation that can occur when using different at-risk population cohorts and geographic scales to determine the incidence risk and to investigate evidence of clustering. Traditionally, incidence risk has been reported at the county level by public health officials, though with focal diseases, this large geographic scale may mask the underlying disease patterns at lower geographic scales. Even so, our findings of a mean incidence risk of 57.59 per 100,000 persons was much higher than those reported by studies in West Virginia, Wisconsin, and Minnesota for the 0-15 year olds, where the incidence risk ranged from 20 to 31 per 100,000 persons at the county level [10,15]. The reason for this higher mean incidence risk is unclear, but could be a result of increased risk of disease transmission, higher rates of diagnosis due to increased awareness of the disease as well as higher and better rates of reporting. This also raises concerns about the problem of under-diagnosis and/or underreporting of severe LACV cases to the health surveillance system.

As expected the higher incidence risks were observed in the younger at-risk age cohorts. Moreover, the differences in incidence risks between the county and the census tract spatial scales were startling. The highest incidence risk in the 0-15 age cohort at the

Table 5. At Risk Populations, Significant Clusters, and the Measures of Incidence Risk at the Census Tract Level for those Census Tracts Displaying Evidence of Spatial Clustering.

At Risk Population	Significant High Clusters*	Incidence Risk per 100,000 persons			
		Median	Range	Mean	Variance
Total population	12	33.20	15.22 – 133.33	46.27	1344.76
0-18 year old population cohort	12	157.48	59.35 – 547.05	204.79	20895.40
0-15 year old population cohort	12	177.62	67.98 – 673.85	235.22	30889.71

*p <0.05

county level was 226.35 per 100,000 persons and was as high as 673.85 per 100,000 persons at the census tract level. When the incidence risk was calculated for only those census tracts within the significant clusters the median incidence risk changed from 126.74 cases per 100,000 persons to 177.62 per 100,000 persons. This information is very useful for public health efforts to control disease as it provides health professionals with precise information regarding the geographic locations and age cohorts to target control strategies and limited resources.

These findings highlight the focal nature of this virus at levels lower than the county level. Although most health reports have always reported health outcomes at the county level, the results from our study indicate that the census tract may be a better level for reporting results as it would provide more precise information regarding high risk populations. Additionally, our findings seem to suggest that there has been a significant increase in disease risk and to our knowledge the highest incidence risk as of yet reported in the literature.

When combined with maps of incidence risk, the use of spatial statistics allows public health professionals to target their limited resources for disease control to active hot-spots of infection (disease). The results from our cluster analyses showed only one significantly high cluster of risk at the county level using the total at risk population and the 0-18 year old population cohort, while no significantly high risk clusters were detected using the 0-15 year old population cohort. This finding highlights the variability that the at-risk populations can have on the spatial analyses, and the need for choosing the appropriate at-risk population and geographic scale for analyses.

At the census tract level, all the at-risk populations had statistically significant geographic hot-spots which occurred in two counties. Though these analyses detected clusters using all population cohorts at the census tract level it should be cautioned that in other regions only the analysis including 0-18 and 0-15 year old age cohorts detected evidence of spatial clustering at the census tract level, highlighting the variability of risk when different age cohorts are studied. Ultimately, the use a lower geographic level allows for the detection of clusters that would be missed at higher levels. These results underscore the need to perform spatial analysis at the lowest possible level to ensure that the underlying spatial disease pattern is not missed.

There are some limitations associated with this data and the methodology used in this study. The probable and confirmed cases were reported through a passive surveillance system. These systems are prone to under-reporting as only the most severe cases typically present for treatment. Notwithstanding this limitation we feel that these cases form the general picture of disease reporting in this region. A downside to the use of the LISA in spatial analyses is the issue of multiple comparisons, which in turn increase type I error rates. These were not adjusted for because such adjustments for type I errors would result in increases in type II errors [159,169]. Thus, such adjustments would in turn lead to a reduction in our test's power to detect truly significant clusters as observed by Rothman and others [169].

Identified patterns in the geographic distribution of disease incidence is useful both to help target areas and populations for intervention purposes and for the allocation of funding for research of disease control. In the United States, Public Health Agencies typically report the incidence at the county level. This is true for many arthropod-borne

viruses (arboviruses) and other diseases reported to the Centers for Disease Control and Prevention [153]. However, our findings indicate that the calculation and reporting of incidence risk at these larger geographic levels (ie county) may lead to a distortion of the underlying spatial patterns of disease risk. This would be most apparent in focal diseases involving a small number of cases and affecting only a sub-set of the general population.

Our findings both reaffirm and highlight the need for the use of the appropriate at-risk population and geographic levels of analysis and reporting of incidence risk involving focal diseases affecting only a sub-set of the population. The 0-15 year old population cohort would provide a more realistic representation of true risk for this virus, when reported at the census tract level. We believe that the continued use of the most appropriate geographic level of geography and at-risk population for LACV infections and other diseases will allow public health officials to better target limited resources for intervention strategies to those areas of highest risk.

CHAPTER V

The spatial epidemiology, clinical presentation, and environmental factors influencing the transmission of La Crosse virus infections in West Virginia, 2003-2007.

**The spatial epidemiology, clinical presentation, and environmental risk factors
influencing the transmission of La Crosse virus infections in West Virginia, 2003-
2007.**

Abstract

The objective of this study was to investigate and describe the spatial epidemiology, clinical presentation, and environmental risk factors associated with La Crosse virus (LACV) infections reported in West Virginia. Descriptive and cluster analyses were performed on probable and confirmed LACV infection cases reported to the West Virginia Department of Health and Humans Resources from 2003 to 2007. Significant ($p < 0.05$) high risk clusters were detected at both the county ($n = 4$) and at the census tract ($n = 30$) levels by global and local spatial statistics. The county level incidence risk for those counties in high risk clusters ranged from between 40.21 to 166.82 cases per 100,000 persons (median 83.16), and between 61.73 to 505.91 cases per 100,000 persons (median 156.50) for those census tracts located in high risk clusters. Cases ranged in age from 0.42 to 54 years, with a majority (84 percent) occurring in children 15 years and younger. Most cases presented during July (28 percent), August (38 percent), and September (17 percent), in the southern region of the state. The most commonly reported symptoms were fever, vomiting, photophobia, and nausea. Clinically reported infections presented as meningitis (41 percent) and encephalitis (40 percent). A case fatality rate was reported in 3 percent of patients. The most commonly observed environmental variables at case residences were the presence of a wooded area (91 percent) and the presence of containers (70 percent). The presence of standing water was observed at almost half of the cases sites (49 percent), and the majority of case sites (76 percent) were located within 45.6 m (149 ft) of a wooded area. Our findings of a higher than expected case-fatality rate and the identification of those areas of highest risk will be useful for guiding future research and intervention efforts.

Introduction

La Crosse virus (LACV) is a member of the genus *Orthobunyavirus* family Bunyaviridae, and is the causative agent of LACV infections. LACV was first isolated from the brain of a deceased pediatric patient who died of encephalitis in 1964 [1], and is one of the most common causes of pediatric arboviral encephalitis in the United States [2,3]. It is transmitted to humans through the bite of infective mosquitoes, the primary vector being the eastern tree-hole mosquito, *Aedes triseriatus* [20]. The virus is maintained in a complex cycle involving both horizontal and vertical transmission in mosquitoes and as well as amplification in squirrel hosts [21,22,27,28] and is typically associated with areas of dense vegetation and/or stands of hardwood trees [11,16,116]. The traditional focus of viral transmission has been in the upper-midwestern United States [2,4,5], but more recently LACV infections have been seen as an emerging disease in West Virginia [8,9]. The majority of cases occur in children under 15 years of age [4,5,29,118], with an average of 100 nationally reported cases per year, though some estimates range much higher [13]. The true incidence of LACV infections are unknown as they are under diagnosed and underreported [6].

From 1964 to 1986, there were 15 reported cases of LACV infections in West Virginia, or 0.87 percent of nationally reported cases. An outbreak LACV infections at a pediatric referral center in 1987 led to active surveillance for LACV in 15 counties, in turn leading to the detection of 19 cases of LACV infections [10]. Eleven of the patients had encephalitis, 4 with meningitis, and 4 had meningo-encephalitis. The ages of patients ranged from 1 to 14 years of age and all fell within the traditional age range of confirmed cases [2,5,42]. Males were found to be more likely infected than females, the ratio being

3.8:1 respectively. Ninety-five percent of the patients were hospitalized and one died. This marked a substantial increase in LACV infections in the state and led to increased surveillance and research activities. From 1987 to 2007, West Virginia reported 555 cases of LAC encephalitis to the CDC, accounting for 31.4 percent of the total cases reported in the United States (Centers for Disease Control, unpublished data).

Following this initial outbreak a case-control study was undertaken to determine the effects of tree holes, artificial containers, and environmental and behavioral factors influencing the transmission of LACV in West Virginia [30]. Cases were matched by age, sex, and geographic location with two controls. A slight increase in disease risk (OR < 2.0) was found for an increased time spent outdoors, the non-use of insect repellent, the non-use of air conditioning, a lack of screened windows, and the non-use of protective clothing. The presence of tree holes near a residence was found to significantly increase the risk of transmission (OR = 8.5 for ≥ 1 tree hole vs. 0 tree holes). Factors found to not statistically significantly increase the risk of disease transmission were artificial containers (OR = 4.1 for ≥ 6 containers vs. 0 – 5 containers), discarded tires (OR = 3.2 for ≥ 10 tires vs. 0 – 9 tires), and the proximity to forest edge (OR = 3.2 for 0 – 14.9 m vs. ≥ 14.9 m). The authors concluded that though these factors were not statistically significant they could still result in increased disease transmission not detected due to limitations of the study.

We conducted both a spatial and descriptive analyses of LACV infections reported in West Virginia from 2003-2007 in an effort to determine those areas at the highest risk for infections, assess the clinical presentation of infections, and to determine

the environmental risk factors of case sites, in an effort to guide intervention strategies by public health officials.

Methodology

Case data. Probable and confirmed cases of LACV infections were collected through passive surveillance from 2003 to 2007, and were obtained from the West Virginia Department of Health and Human Resources. Personal identifiers of patients were deleted before the data were released for the study. Clinical data were available for 96 cases, and data on the location of residence were available for 68 patients. Ages ranged from 0 to 15 years old.

Confirmed cases of LACV infections met both the clinical and laboratory requirements set by the Centers for Disease Control and Prevention's case definition for neuroinvasive domestic arboviral diseases [132,153]. Those cases that met the clinical definition and the initial antibody screening detecting virus specific antibodies, were classified as probable cases.

Population, geographic, and environmental data. As the majority of LACV infections are pediatric [4,5,29,118], it was deemed appropriate to use the 0-15 year old population for the calculation of incidence risk for these infections. The decennial 2000 United States Census was used to calculate the 0 to 15 year old population for each county and census tract. Geographic boundary files were downloaded from the United States Census, TIGER, Geodatabase [156], and used for all cartographic displays. Environmental officers of the West Virginia Department of Health and Human Resources conducted environmental assessments at both probable and confirmed patients residences during the study period.

Statistical and geographic analyses. The incidence risks were calculated at both the county and the census tract spatial scales and spatial analyses was performed on 68 cases 0 to 15 years old for which location data were available. The incidence risks were calculated for all counties in the study area ($n = 55$), for counties reporting cases ($n = 18$), and for all census tracts in the study area ($n = 466$), and for census tracts reporting cases ($n = 50$). Incidence risk was expressed as the number of cases per 100,000 persons.

Descriptive analyses and the calculation of incidence risk were performed using STATA 10.0 [157]. We adjusted for the high variances resulting from a small number of cases reported in some counties and census tracts by smoothing the risk using spatial empirical Bayesian (SEB) smoothing [158,159,160,161]. This technique was implemented in GeoDa [164] using inverse distance spatial weights. The use of SEB smoothing increased the overall visual perception of spatial patterns when compared with unsmoothed maps of incidence risks.

Evidence of spatial clustering was assessed by the Global Moran's I [162] and the Moran Local Indicators of Spatial Association (LISA) [163], implemented in GeoDa Version 0.95i [164], using inverse distance spatial weights. Statistical significance was tested using 9999 permutations. Cartographic displays were made using ArcView GIS 9.2 [165].

Results

Spatial analyses. The highest incidence risks were observed at the census tract level (Table 6). The incidence risk for counties reporting cases ranged from 7.20 to

Table 6. Comparisons of the Incidence Risk of La Crosse Infections for West Virginia County and Census Tracts

Geographic Risk Level	Obs.	Incidence Risk per 100,000 persons			
		Median	Range	Mean	Variance
County (Entire Study Area)	55	0	0-166.82	15.48	1057.69
County (Only Those Reporting Cases)	18	32.07	7.20-166.82	47.29	1766.50
County (Only those in High-Risk Clusters)	4	83.16	40.21-166.82	93.35	2871.96
Census tract (Entire Study Area)	466	0	0-505.90	17.39	3158.20
Census tract (Only Those Reporting Cases)	50	150.04	42.41-505.90	162.08	6042.14
Census tract (Only those in High-Risk Clusters)	30	156.50	61.73-505.91	180.66	7604.50

Obs = Observations, and refer to the number of counties or census tracts used for each analysis.

166.82 per 100,000 persons (median 32.07), and from 42.41 to 505.90 per 100,000 persons for those census tracts reporting cases.

The highest unsmoothed risks were observed in the south-central region of the state at both the county and census tract levels (Figure 7). Visually, the spatial patterns of SEB smoothed risk at the county and census tract levels were more evident and followed similar patterns as the unsmoothed risks (Figure 7), though only some of the census tracts within the high-risks counties, had high-risks of infection.

The Global Morans I detected significant clustering at the county and census tracts with values of 0.4986 ($p = 0.0001$) and 0.2935 ($p = 0.0001$), respectively. The LISA detected significant ($p < 0.05$) high risk clusters at both the county ($n = 4$) and at the census tract ($n = 30$) levels (Figure 8). At the county level significant high risk clusters were detected in Fayette, Raleigh, Nicholas, and Wyoming counties. Significant high-risk clusters were detected at the census tract level within the counties of Fayette (9 census tracts), Mercer (9 census tracts), Raleigh (8 census tracts), Nicholas (2 census tracts), Kanawha (1 census tract), and Wyoming (1 census tract). The county level incidence risk for those counties in high risk clusters ranged from between 40.21 to 166.82 cases per 100,000 persons (median 83.2), and between 61.73 to 505.91 cases per 100,000 persons (median 156.5) for those census tracts in high risk clusters (Table 6).

Clinical. Cases ranged in age from 0.42 to 54 years, with a mean age of 10.8 (SD 10.3) years, of which 84 percent occurred in children under the age of 15 (Table 7). The vast majority of cases presented during July (28 percent), August (38 percent), and September (17 percent), in the southern region of the state.

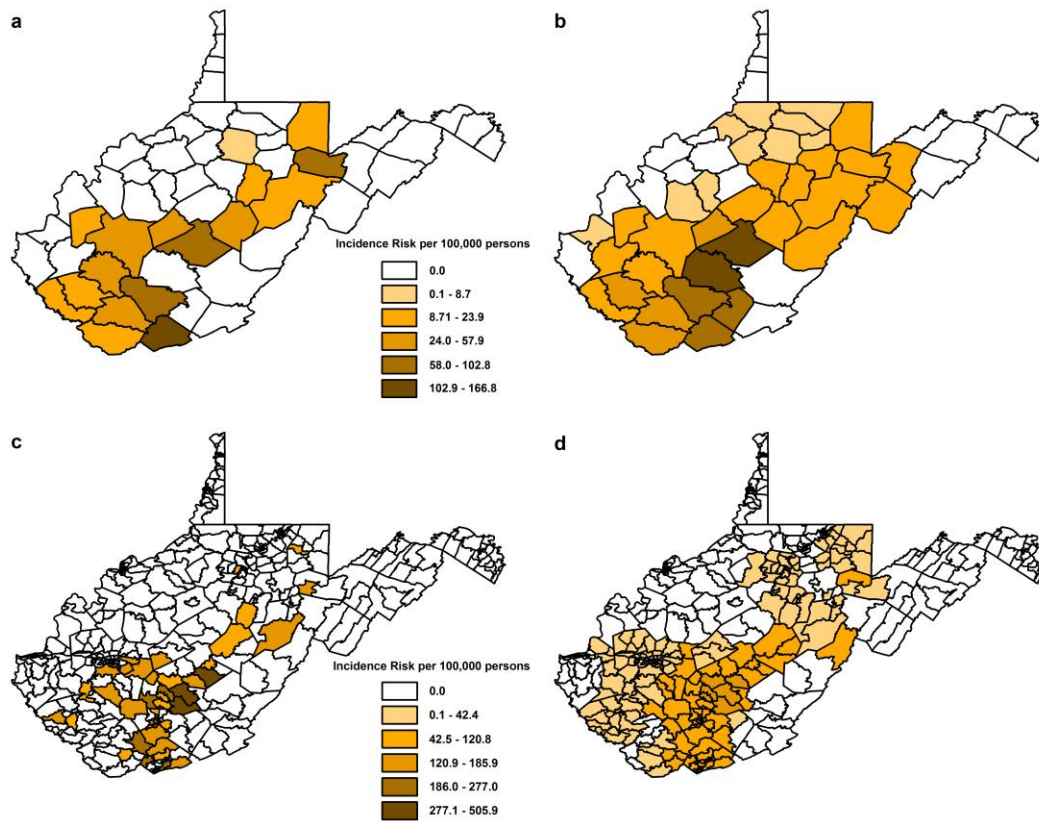


Figure 7. The unsmoothed and smoothed incidence risk at the county and census tract level.

The distribution of unsmoothed risk of La Crosse infections at the county (a) and the census tract level (c) for West Virginia. The distribution of spatial empirical Bayesian smoothed risk for La Crosse infections in the West Virginia at the county (b) and the census tract level (d).

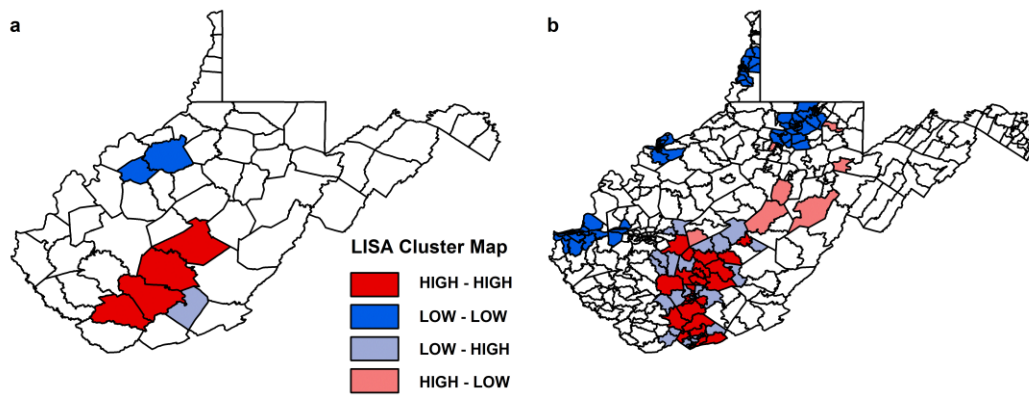


Figure 8. The spatial clustering of La Crosse virus infections at the county and census tract levels. These maps show the significant high clustering (HIGH-HIGH) of La Crosse infections at the county level (a) and at the census tract level (b) detected by the Moran's I Local Indicators of Spatial Autocorrelation.

Table 7. Characteristics of Cases of La Crosse
Infections Reported to the West Virginia
Department of Health and Human Resources, 2003-
2007

Variable	Number (%)
Sex	
Male	58 (60)
Female	38 (39)
Age	
0.1 - 0.9 yr	1 (1)
1 - 5 yr	27 (28)
6 - 10 yr	38 (40)
11 - 15 yr	15 (16)
16 - 20 yr	6 (6)
≥ 21 yr	9 (9)
Month of presentation	
May	1 (1)
June	8 (8)
July	27 (28)
August	36 (38)
September	16 (17)
October	8 (8)
Reported Symptoms	
Fever	73 (76)
Vomiting	72 (75)
Elevated CFS WBC	68 (71)
Photophobia	50 (52)
Nausea	48 (50)

Table 7 Continued. Characteristics of Cases of La Crosse Infections Reported to the West Virginia Department of Health and Human Resources, 2003 to 2007

Weakness	40 (42)
Meningitis	39 (41)
Encephalitis	38 (40)
Stiff Neck	32 (33)
Confusion	32 (33)
Seizures	23 (24)
Elevated CSF protein	24 (25)
Myalgia	11 (11)
Rash	7 (7)
Arthralgia	7 (7)
Coma	4 (4)
Died	
Male	2 (2)
Female	1 (1)

The most common symptoms reported were fever, vomiting, photophobia, and nausea (Table 7). Meningitis and encephalitis were reported in 41 and 40 percent of cases respectively. Other reported symptoms were weakness (42 percent), stiff neck and confusion (33 percent), seizures (24 percent), and coma (4 percent). A case fatality rate was reported in 3 percent of patients.

Elevated levels of white blood cell (WBC) counts in cerebral spinal fluid (CSF) were reported in 73 cases (76 percent), of which 68 cases had counts ranging from 10 to 670 per mm³, with a mean count of 160.9 per mm³ (SD 153.8). Health care providers reported either CSF protein levels and/or the presence of elevated CSF protein levels. CSF protein levels were available for only 27 cases, and ranged from 24 to 359 mg/dl, with a mean level of 64.4 mg/dl (SD 61.6). Elevated CSF protein levels were reported in 24 cases (25 percent). Fever was present in all cases for which temperature was reported (n = 54), with a mean value of 39.4°C (SD 0.66) and ranged from 38.3°C to 40.6°C (100.9°F to 105°F).

Environmental. The most commonly observed environmental variables at case residences were the presence of a wooded area (91 percent) and the presence of containers (70 percent) (Table 8). The presence of standing water was observed at almost half of the cases residences (49 percent), and the majority of cases residences (76 percent) were within 45.6 m (149 ft) of a wooded area (Table 9).

Discussion

The highest incidence risks were observed in the south-central region of the state, as reported by previous studies [30,171]. The reason for this increase in risk is not

Table 8. The Observed and Reported Presence of Environmental Risk Factors at 97 Cases of La Crosse Infections Reported to the West Virginia Department of Health and Human Resources, 2003-2007

Variable	Present (%)	Missing Data
Containers*	60 (70)	11
Other containers [†]	53 (62)	12
Tires	33 (37)	15
Tarps	32 (41)	19
Standing water	41 (49)	13
Wooded area	81 (91)	8
Hardwood	68 (93)	24
Evergreen	35 (70)	47

*Containers, were all containers including: other containers, tires, and tarps. These variables were cross-referenced with one another for each case to determine the presence of at least one variable at each case site.

Table 9. The Numbers of Observed and Reported Potential Larval Habitats Present at 97 Cases of La Crosse Infections Reported to the West Virginia Department of Health and Human Resources, 2003-2007

Variable	Number (%)	Missing Data
No. of tires		20
0	49 (64)	
1-9	21 (27)	
≥ 10	7 (9)	
No. of tarps		31
0	46 (70)	
1-10	21 (32)	
No. of other types of containers [†]		23
0	19 (26)	
1-5	29 (39)	
6-15	10 (14)	
≥ 16	3 (4)	
Distance (meters) from residence to wooded area		23
0-14.9 (0-49 ft)	35 (57)	
15-45.6 (50-149 ft)	21 (34)	
≥ 45.7 (≥ 150 ft)	6 (10)	

[†]Other types of containers were those containers not including tires and/or tarps.

understood, but could be due to a variety of factors including increased surveillance, diagnosis, and reporting of infections.

As anticipated, the highest incidence risks were observed at the census tract level rather than the county level (Table 7). Our results further demonstrate that the census tract level would be the preferred geographic level for reporting cases and calculating incidence risk for focal diseases (Haddow in Review). Further, we recommend reporting the range of incidence risk rather than only the mean incidence risk within those census tracts reporting cases. Such reporting would provide a more representative picture of the burden of disease risk.

Significant high disease clustering was observed at both the county and census tract levels and was detected in the south-central region of the state. Four clusters of disease were detected at the county level, while there were 30 clusters observed at the census tract level, which were located within 6 counties. These results demonstrate the usefulness of using the lower geographic levels and spatial statistics for the detection of disease clusters of focal diseases.

A 2003 study reported the occurrence of mosquito larvae inhabiting tires in different geographic regions (western, central, and eastern) of the state [19] and provides the most recent account of the biology and distribution of vector species in West Virginia. The results of this study inferred a shift in vector abundance in West Virginia, with *Ae. japonicus* being collected more frequently than *Ae. triseriatus* in all geographic regions of West Virginia, thus increasing the likelihood that this species could be an accessory vector of LACV in West Virginia. Further findings that *Ae. albopictus* were collected at significantly lower ($p < 0.05$) numbers than *Ae. triseriatus* at both

peridomestic and non-peridomestic sites in the central and eastern regions of the state could indicate that *Ae. albopictus* is not playing an important role as accessory vector of LACV in West Virginia [9,10,30], as these regions experienced the highest incidence risks and were also the areas of the significantly high disease clusters.

In agreement with previous work [29], our study shows that the majority of reported LACV infections occur in male children 15 years and younger during the summer months and display fever, headache, vomiting, and mental status changes. These cases have traditionally proven difficult to distinguish from Herpes simplex meningoencephalitis [29,138]. The elevated CSF WBC counts found in our study remain a diagnostic tool, though the results should be interpreted with caution as patients frequently demonstrate a predominance of Polymorphonuclear Leukocytes on their peripheral blood smear and CSF [29]. Such findings may suggest a bacterial infection, and may result in a possible delay in the diagnosis of this viral illness [29]. A troubling discovery in this study is the continued high case fatality rate (3 percent) in West Virginia [30], compared to a much lower case fatality rate (1.5 percent) reported during the same time period for the rest of the United States [30,153]. The reason for this higher case fatality rate in West Virginia is unclear, but may indicate the possibility of a more virulent strain(s) of the virus circulating in this region [67].

The presence of a wooded area with tree-holes and containers are widely acknowledged as risk factors for LACV [2,4,14,30,148,151]. We found that a majority of cases, 76 percent, were within 45.6 m (149 ft) of a wooded area, and 70 percent had observed containers present at case residences. The presence of water holding containers remains a high source of larval habitat at these sites, and of these cases, 37 percent had

observed tires, 37 percent had observed tarps, and 62 percent had the observed presence of other types of containers.

Though we had no control group to compare our findings, the large number of cases with the observed proximity to wooded areas and containers near the case residences are in agreement with the findings from a previous study in this region as a risk factor for LACV [30]. Thus, the elimination of water holding containers and the closure of tree-holes would likely reduce the overall burden of infective mosquitoes and decrease the risk of LACV transmission, as seen elsewhere [148].

Due to the methodology and data employed in our study there are some limitations. Passive surveillance systems are prone to under-reporting/detection of disease, though we feel that the majority of cases suffering from severe illness were diagnosed and reported to state health officials. Clinical data was reported from multiple health care providers to the West Virginia Department of Health and Human Resources, which did not allow for the verification of laboratory results and the diagnosis of the specific clinical presentation for each patient. Another drawback is the issue of multiple comparisons, which can occur when using the LISA statistic for cluster detection, which would increase type I errors. These errors were not adjusted for, as adjustments for type I errors would increase type II errors [159,169], in turn reducing the ability to detect truly significant clusters [169].

During the study period, West Virginia reported the highest incidence risk of LACV infections in the United States, marking a shift from the traditional focus of transmission from the upper Midwestern States to the Appalachian Region. The reason for this shift is unclear, but could be due to a variety of factors including increases and/or

decreases in diagnosis, reporting and prevention. Future case-control studies are needed to determine current risk factors and should include vector and host sampling. In addition virus isolations are needed to compare WV virus strains with virus strains from other regions. Though reporting of the clinical presentation of LACV infections through a passive surveillance system is not without limitations, such reporting will allow for the continued monitoring of trends in the virulence of infections. Our findings demonstrate that south-central West Virginia remains a focus of LACV transmission and highlights the utility of using the combination of incidence risk and spatial statistics to detect areas of high virus transmission. The presence of wooded areas and containers in virus foci remain a risk factor for acquiring LACV. These findings will allow public health officials to target these areas for interventions.

SECTION III: BIOLOGY AND ECOSYSTEM DYNAMICS OF DISEASE VECTORS

CHAPTER VI

The Mosquitoes of eastern Tennessee: Studies on Abundance, Habitat Preferences, and Host-Seeking Behaviors

This chapter is a lightly revised version of a paper by the same name *ACCEPTED* in the *Journal of Vector Ecology* in 2009 by Andrew D. Haddow, Reid R. Gerhardt, Carl J. Jones, and Agricola Odoi:

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My contributions to this paper are 1) substantial contribution to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published.

Abstract

In the last ten years there has been a significant increase in the number of reported cases of La Crosse virus (LACV) infections in eastern Tennessee. The objective of this study was to determine the abundance and habitat preferences of the potential vectors of LACV in this region. Adult host-seeking mosquitoes were collected using CO₂-baited Centers for Disease Control (CDC) light traps and by a series of human-landing catches in eastern Tennessee from 2004 to 2006. A total of 23 species, totaling 4,200 female mosquitoes were collected by CO₂-baited CDC trapping at 10 sites during the study period. *Aedes albopictus* (Skuse) was the most abundant mosquito collected at all sites and vegetation types, with the ratios of total *Ae. albopictus* to *Ae. triseriatus* (Say) females collected being 2.1:1 in 2004, 3.8:1 in 2005, and 4.9:1 in 2007. Ten species of mosquitoes were collected during a series of human-landing catches made at four different sites; one probable and three confirmed case sites of LACV infections, totaling 528 female mosquitoes. *Aedes albopictus* was the most abundant species collected, with the proportion of *Ae. albopictus* to *Ae. triseriatus* females collected being 4:1. *Aedes albopictus* exhibited two clear peaks of “landing” activity, one in the early morning and one in the late afternoon or early evening. Simple and multiple regression analyses of the predictors of the number of mosquitoes collected, showed that populations of *Ae. albopictus* were three times more likely to be collected overall than *Ae. triseriatus*. Species (*Ae. albopictus*), vegetation (residential), and the previous cumulative precipitation for the four weeks prior to collection were significantly ($P < 0.05$) associated with the number of mosquitoes collected by CO₂-baited CDC trapping. *Aedes*

albopictus was also more likely to be collected than *Ae. triseriatus* at confirmed cases of severe LACV infections.

Introduction

La Crosse virus (LACV) is one of most frequently identified arboviral causes of pediatric encephalitis in the United States [2,3]. It has traditionally been associated with the upper-Midwestern United States [4] though it has been reported in other regions [9]; recently as an emerging disease the southeastern United States [6,7,16]. LACV infections became seasonally epidemic in eastern Tennessee in the mid-1990's coinciding with the establishment of *Aedes albopictus* (Skuse) (Gerhardt unpublished). Prior to the mid-1990's, there had been nine reported cases of LACV infections from 1964-1996 in Tennessee [6].

Aedes triseriatus (Say) is considered the primary vector of LACV within its traditional range [2,9,21]. It is typically associated with hardwood forests and is primarily a tree-hole mosquito, but will also oviposit in man-made containers [9]. LACV can be transmitted transovarially and venereally in this species and is able to overwinter in its eggs [4,21,22,26,85]. It is known to feed on a variety of mammals including squirrels, chipmunks, and humans [86]. This species is widely distributed throughout the eastern United States (Darsie & Ward 1981) and has been found at LACV infection case sites in Tennessee [16]. The invasive mosquito *Ae. albopictus* was first collected in the USA in Houston, Texas in 1985 [89] and is believed to have been introduced to the United States through shipments of tires from Asia [90,91]. Since its establishment, *Ae. albopictus* has spread over a large portion of the United States, including Tennessee [92,172]. It is known to feed on a variety of animals including man [93,114]; its larvae

are found in both man-made containers and tree holes [35,91]. *Aedes albopictus* has been shown to be a competent laboratory vector of several viruses, including LAC virus [23], and may be associated with the increase of LACV infections in Tennessee [16,17].

Continued surveillance recently detected the establishment of a second invasive mosquito vector capable of transmitting the virus, *Ae. japonicus* (Theobald) [24,38]. *Aedes japonicus* has been found at case sites of LACV infections in the region, in addition to *Ae. triseriatus* and *Ae. albopictus* [16,17,20,35,36,99].

In 1997, a cluster of 10 cases of LACV infections were detected in eastern Tennessee [6], during which time active surveillance for the virus was initiated by both the University of Tennessee and The Tennessee Department of Health. Surveillance included mosquito trapping at LACV infection case sites [6,16,17,35]. Trapping of mosquitoes at these sites led to the first isolation of LACV from *Ae. albopictus* (TN00-2266) and the first isolation of the virus in Tennessee. This isolation came from a pool of 14 reared females collected as eggs at a confirmed case site (onset date 23 June 1998) in Anderson County, 16 August 1999 [17]. The minimum infection rate for *Ae. albopictus* eggs oviposited at the site was determined to be 6.5/1000 for the isolation week, with a ratio of collected *Ae. albopictus* to *Aedes triseriatus* (Say) of 153:1. This residence was well maintained with an oak and hickory forest located 15 meters from the residence.

To determine the abundance and habitat preferences of the vectors of LACV in eastern Tennessee and to explore the role of *Ae. albopictus* as a potential vector of LACV, weekly seasonal trapping of host-seeking species was initiated in 2004 and continued through 2006. In addition, a series of human-landing catches were conducted in 2004. We present the results of these studies below.

Methodology

Study Areas. Collections took place at 14 sites in four counties in eastern Tennessee (Table 10), an area endemic for LACV transmission [6,7,16]. Sites were classified as either probable sites of LACV infection, confirmed sites of LACV infection, or sites having no previously reported cases of LACV infections, by the Tennessee Department of Health. CO₂-baited CDC trapping took place at 10 sites in Knox County. Knox County has total area of 508.46 square miles and a population density of 752 persons per square mile [173]. Human-landing catches also took place in: Campbell County, total area of 480.07 square miles, and a population density of 83 persons per square mile; Claiborne County, total area of 434.28 square miles, and a population density of 68.8 persons per square mile; and Union County, total area of 223.56 square miles, and a population density of 79.5 persons per square mile [173].

Vegetation. The predominant vegetation species were identified at each collection site for the canopy, intermediate, understory, and ground levels (Table 11). Vegetation types at each site were further characterized using the “*The Forest Cover Types of the United States and Canada*” [174], with the exception of residential and agricultural sites which did not meet the requirements for characterization and were defined by their location within residential or agricultural areas. Vegetation was then classed into four types: northern red-oak, yellow-poplar white-oak, residential, and agricultural (Table 10).

Table 10. Site identification, trapping years, vegetation type, county, catch type and the reporting of La Crosse infection from 2004 to 2006.

Site Identification	Trapping Years	Vegetation Type	County	Catch Type	Reporting of La Crosse infection
1	All	Yellow – poplar – white oak – northern red oak	Knox	CO ₂ -baited CDC trap	Confirmed
2	All	Northern red oak	Knox	CO ₂ -baited CDC trap	No
3	All	Yellow – poplar – white oak – northern red oak	Knox	CO ₂ -baited CDC trap	No
4	2004	Urban – residential	Knox	CO ₂ -baited CDC trap	No
5	All	Yellow – poplar – white oak – northern red oak	Knox	CO ₂ -baited CDC trap	No
6	All	Urban – residential	Knox	CO ₂ -baited CDC trap	No
7	All	White oak – black oak – northern red oak	Knox	CO ₂ -baited CDC trap	Confirmed
8	All	Yellow – poplar – white oak – northern red oak	Knox	CO ₂ -baited CDC trap	Confirmed
9	2005, 2006	Northern red oak	Knox	CO ₂ -baited CDC trap	No
10	2005, 2006	Urban – residential	Knox	CO ₂ -baited CDC trap	No
11	2004	Yellow – poplar – white oak – northern red oak	Campbell	Human landing-catch	Confirmed
12	2004	Agricultural	Claiborne	Human landing-catch	Confirmed
13	2004	Yellow – poplar – white oak – northern red oak	Campbell	Human landing-catch	Confirmed
14	2004	Yellow – poplar – white oak – northern red oak	Union	Human landing-catch	Probable

Table 11. Stratification of vegetation by site.

Vegetation Species	Stratification of Vegetation Dominance			
	Canopy	Intermediate	Understory	Ground
<i>Albizia julibrissin</i> (mimosa)			4, 13	
<i>A. saccharum</i> (sugar maple)	4, 6, 7, 8	5, 6, 7, 8	1, 2, 3, 6, 7, 8, 12, 23, 14	
<i>Carpinus caroliniana</i> (American hornbeam)		3	7, 8	
<i>Carya cordiformis</i> (bitternut hickory)	1, 2, 7, 8, 10	7		
<i>C. glabra</i> (Pignut hickory)	2	2		
<i>C. ovata</i> (shagbark hickory)	9			
<i>Celtis occidentalis</i> (hackberry)	4, 9	9		
<i>Cercis canadensis</i> (eastern redbud)		8, 13	8	
<i>Cornus florida</i> (flowering dogwood)		1, 5, 14	7, 13	
<i>Fagus grandifolia</i> (American beech)			9, 11	
<i>Hedera helix</i> (English ivy)				5, 6
<i>Juniperus virginiana</i> (eastern red cedar)	4, 6	3	10, 12	
<i>Ligustrum sp.</i> (privet)			1, 3, 4, 5, 6, 7, 9, 11, 14	9, 11, 13
<i>Liriodendron tulipifera</i> (yellow poplar)	1, 3, 5, 8, 11, 13, 14	5, 11, 13		
<i>Liriope sp.</i> (lily turf)				3, 10
<i>Lonchocera tartarica</i> (honeysuckle)		13	2, 3, 9, 11	
<i>L. japonica</i> (Japanese honeysuckle)			3, 4, 5, 6	1, 2, 4, 5, 6, 7, 10, 13
<i>Oxydendrum arboreum</i> (sourwood)		1		
<i>Pinus strobus</i> (eastern white pine)	5, 6, 9, 10	14		

Table 11 *Continued*. Stratification of vegetation by site.

<i>Phyllostachys</i> sp. (bamboo)	5	5	
<i>Poa</i> sp. (bluegrass)			4, 5, 9, 10, 11, 12, 14
<i>Prunus serotina</i> (black cherry)	3, 4, 10	8	
<i>Quercus alba</i> (white oak)	3, 7, 11, 13, 14	3, 5, 11, 13, 14	1, 6, 8, 11
<i>Q. falcata</i> (southern red oak)	7, 10		1
<i>Q. prinus</i> (chestnut oak)	2	8, 9	1, 14
<i>Q. rubra</i> (red oak)	2, 3, 5, 8, 9, 10, 11, 12, 14	8	6, 9
<i>Ribes</i> sp. (gooseberry)			2, 3, 7, 8, 11, 13
<i>Rosa</i> sp. (rose)			2, 3
<i>Tilia americana</i> (American basswood)	2, 5, 8	5	

Sampling techniques. Adult host-seeking mosquitoes were collected using CO₂-baited Centers for Disease Control (CDC) light traps (John W. Hock Company, Gainesville, FL) with the lights removed. Trapping methodology has been described elsewhere [175], briefly each trap had ~ 2 kg of dry ice placed inside a modified Igloo[®] cooler (John W. Hock Company, Gainesville, FL) suspended above the trap allowing for sublimation of the dry ice directly above the traps location. Traps were suspended approximately 1.0 m off the ground, at the same location at each site for each trapping year. Each site had one trap in operation for 24 H time period, once a week, from epidemiological week 23 through week 40 for each trapping year. Adult mosquitoes were identified by species and sex [87].

One human-landing catch took place at each of three confirmed and one probable case site of LACV transmission upon notification by the Tennessee Department of Health and release of confidentiality by the patient's guardians. Cases were deemed to be confirmed if they met both the clinical and laboratory case definition set by the CDC, and cases were deemed to be probable upon the results of acute serum testing for IgM and IgG antibodies for LACV [132]. Catches were made seated by the same individual (A.D.H.), wearing the same clothes, in an effort to standardize any variables that might affect host attractiveness. All collections took place from 30 minutes pre-civil twilight to 30 minutes post-civil twilight during a one-day period. Two different collection regimes were used. At sites 11 (20 June 2004) and 12 (2 July 2004) each collection period lasted 15 minutes. Trapping took place at four locations each 20 meters apart around the perimeter of the residence, with each collection period taking place consecutively after the previous collection from the start until the conclusion of trapping. At sites 13 (15

July 2004) and 14 (11 September 2004) a collection period lasted 30 minutes, and was made at two locations at each residence 20 meters apart. Again each collection period took place consecutively following the previous collection period from the start until the conclusion of trapping. Adult mosquitoes were collected in 15 mL Nunc EZFlip tubes (Nalge Nunc International, Rochester, NY). Collected mosquitoes were stored at -20°C until taken to the laboratory where they were identified by species and sex [87].

Weather data. Weather data was acquired from the National Climatic Data Center [176] for CDC trapping of adult host-seeking mosquitoes in Knox County, TN. The nearest weather station to Knox County, TN, having continuous data available for the study period was used (COOPID 4049950, 35°49N / 83°59W, elevation 293.2 meters above sea level). COOPID 4049950 is located on the southern border of Knox County, TN and Blount County, TN. All sites in Knox County were less than 40 km from COOPID 4049950. Daily temperatures and precipitation were converted to weekly mean temperatures and cumulative weekly precipitation totals for the study period (Figure 9). Temperature and relative humidity for the period of each human-landing catch was collected at each site using a Kestrel 3000 Pocket Weather Meter (Nielsen Kellerman, Chester, PA). Temperature and relative humidity readings were averaged to obtain mean readings for each two-hour period (Figure 10).

Statistical analyses. All statistical analyses were performed using STATA 10.0 (STATA Corp. College Station, TX). Graphs were generated using DataGraph 10.5 Beta (Visual Data Tools, Inc., Chapel Hill, NC). Simple regression analyses were used to assess the univariate associations between the numbers of adult female mosquitoes of a particular species (*Ae. albopictus* and *Ae. triseriatus*) collected and each of the suspected

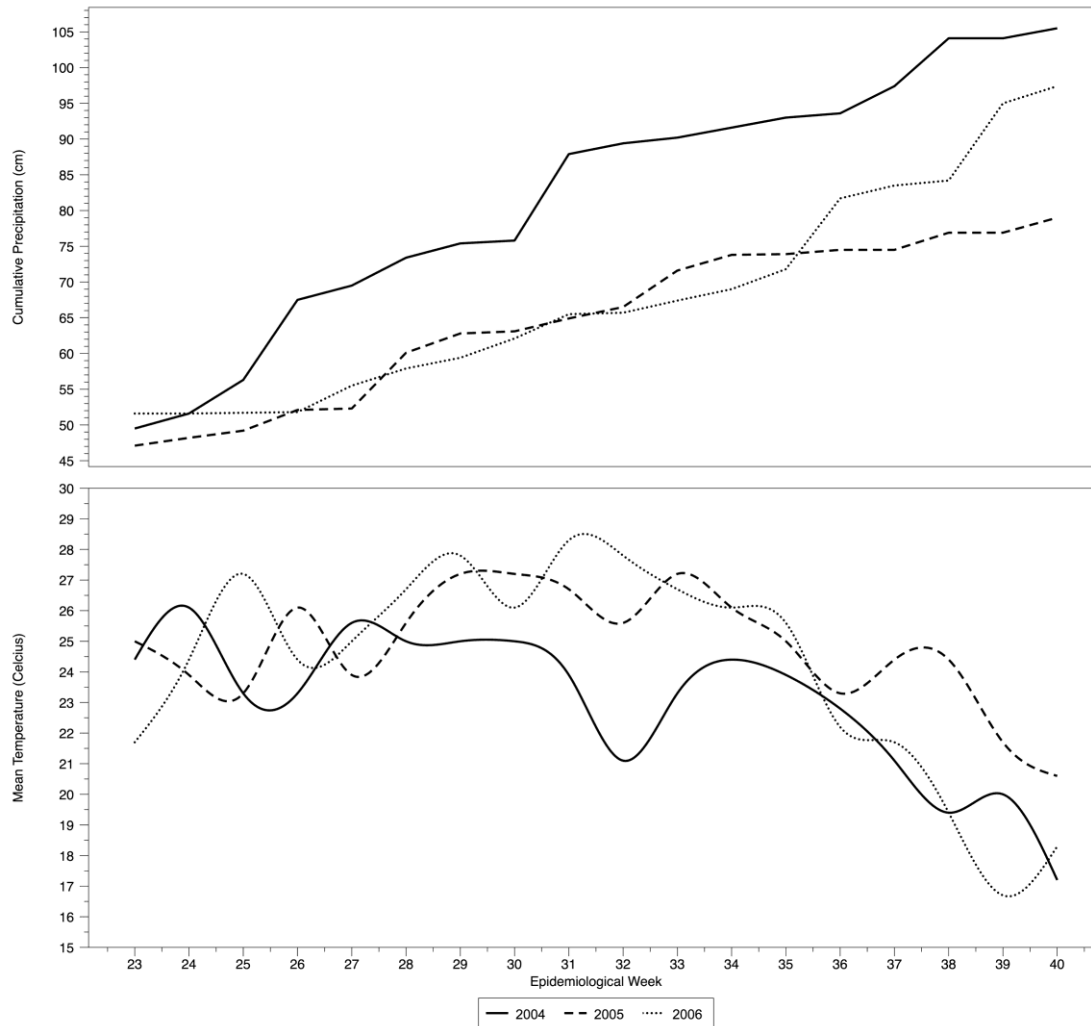


Figure 9. The cumulative precipitation (top) and the mean temperature (bottom) for Knox County, TN 2004 to 2006.

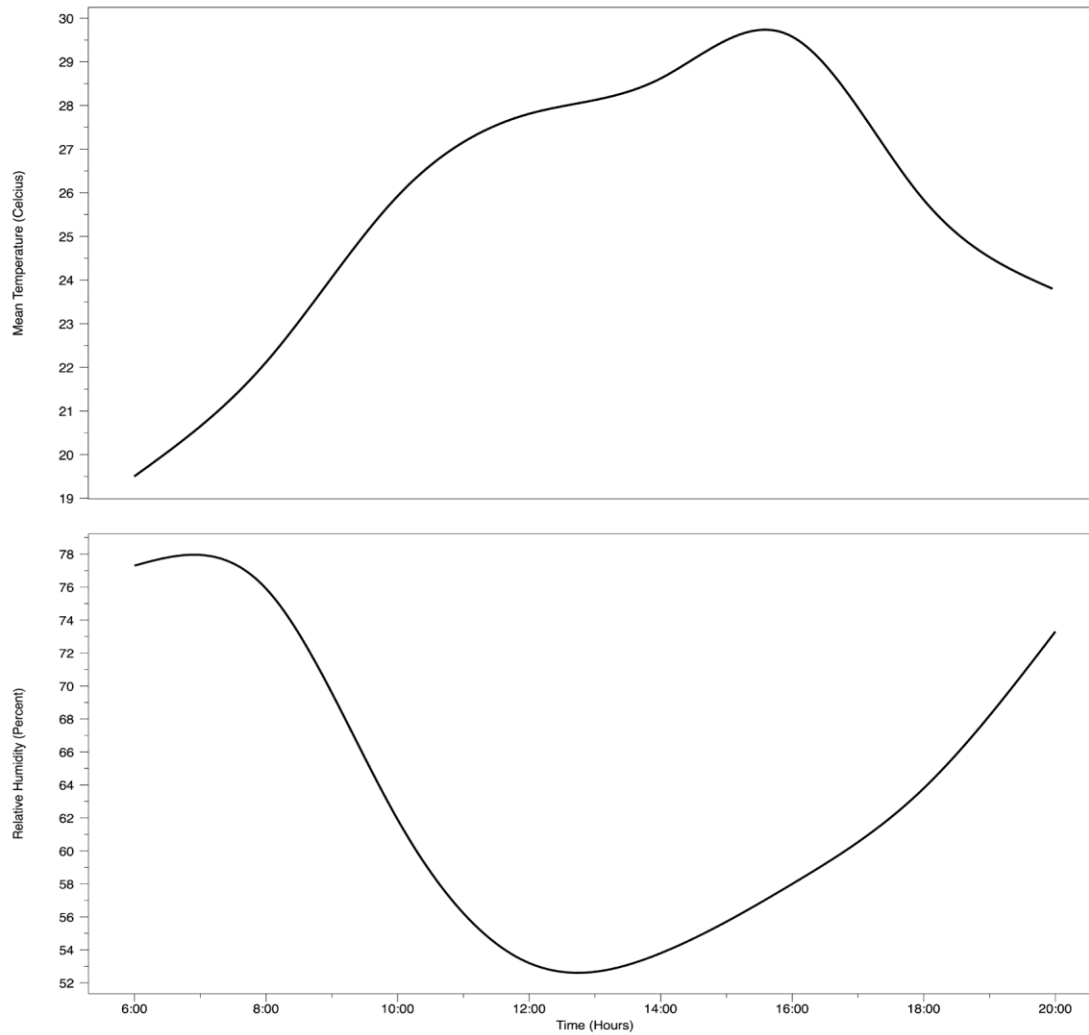


Figure 10. The mean temperature (top) and mean relative humidity (bottom) at a series of human-landing catches*.

*Mean temperature and mean relative humidity were calculated from the three separate human-landing catches where *Aedes albopictus* and *Aedes triseriatus* were collected.

predictors: species, vegetation type, previous reporting of LACV infections, temperature for the week of collection (mean, min, max), precipitation for the week of collection, cumulative precipitation for the four weeks prior to each collection (Table 12).

Variables that had significant univariate associations with the number of mosquitoes were further investigated using a multiple regression analysis. These were: species (*Ae. albopictus* and *Ae. triseriatus*), vegetation type, cumulative precipitation for the four weeks prior to each collection. Only variables that were significant ($P < 0.05$) in the multiple regression analyses were retained in the final model.

Results

Adult sampling. During the three-year study period 23 species totaling 4,200 female mosquitoes were collected using CO₂-baited CDC traps (Table 13). The three most frequently collected species during each of the three collection years were *Ae. albopictus*, *Ae. triseriatus*, and *Ae. vexans* (Meigen). *Aedes albopictus* was the most abundant species collected during the study period, at all vegetation types, and all study sites. *Aedes triseriatus* was the second most abundant species collected in 2004 and 2006, and third most abundant in 2005. *Aedes vexans* was the third most abundant species in 2004 and 2006, and the second most abundant species 2005. The ratios of total *Ae. albopictus* to *Ae. triseriatus* females collected at all sites was 2.1:1 in 2004, 3.8:1 in 2005, and 4.9:1 in 2007 (Figure 15).

Ten species of mosquitoes were collected by human land-catches totaling 528 female mosquitoes (Table 14). *Aedes albopictus* was the most abundant species collected. *Aedes albopictus* and *Ae. triseriatus* were collected from sites 11, 13, and 14. The ratios of total *Ae. albopictus* to *Ae. triseriatus* females collected was 4:1 (Table 15).

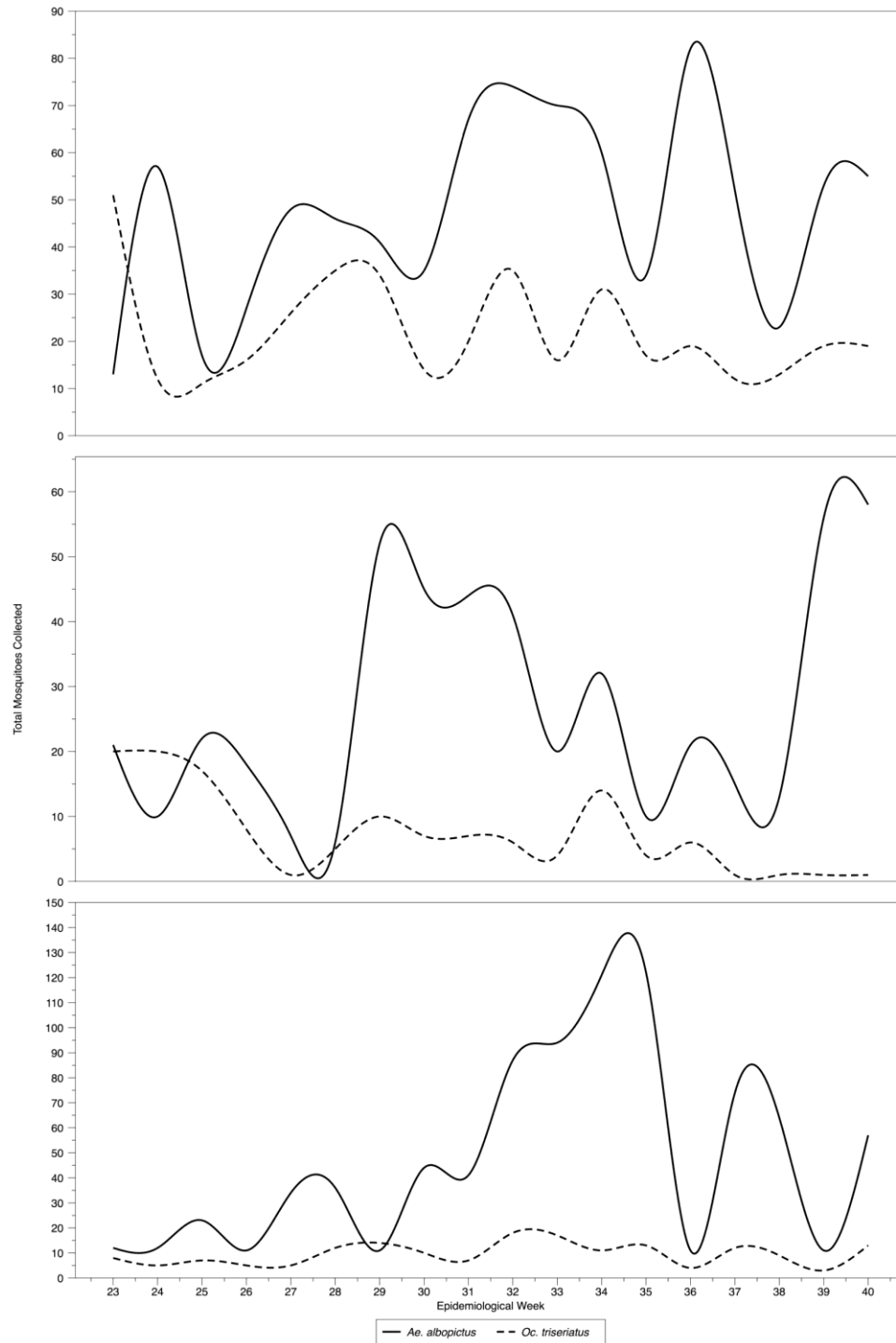


Figure 11. The total number of *Aedes albopictus* and *Aedes triseriatus* collected by CO₂-baited CDC trapping for 2004 (top), 2005 (middle), and 2006 (bottom).

Table 12. Variables used in the analysis to predict mosquito abundance.

Variable	Variable description	Coding	Variable type	Unit of measurement	Dependent vs. independent
Number of mosquitoes	Total number of mosquitoes collected	Numeric	Continuous	Count	Dependent
Species	Species of mosquitoes collected	1 = <i>Aedes albopictus</i> 2 = <i>Aedes triseriatus</i>	Categorical	None	Independent
Vegetation	Vegetation type	1 = Yellow-poplar-white oak-northern red oak 2 = Northern red oak 3 = Residential	Categorical	None	Independent
Precipitation	Cumulative precipitation	Numeric	Continuous	cm	Independent

Table 13. Collected female mosquito totals and percentage yield by year, by CO₂-baited CDC trapping.

2004			2005			2006		
Species	No. of mosquitoes	%	Species	No. of mosquitoes	%	Species	No. of mosquitoes	%
<i>Ae. albopictus</i>	857	49.0	<i>Ae. albopictus</i>	488	47.0	<i>Ae. albopictus</i>	865	59.0
<i>Ae. triseriatus</i>	401	23.0	<i>Ae. vexans</i>	168	16.0	<i>Ae. triseriatus</i>	172	12.0
<i>Ae. vexans</i>	148	8.4	<i>Ae. triseriatus</i>	132	13.0	<i>Ae. vexans</i>	151	10.0
<i>Aedes spp.</i>	112	6.4	<i>An. punctipennis</i>	114	11.0	<i>An. punctipennis</i>	109	7.5
<i>Culex spp.</i>	69	3.9	<i>Aedes spp.</i>	40	3.8	<i>Culex spp.</i>	77	5.3
<i>An. punctipennis</i>	49	2.8	<i>Cx. pipiens</i>	36	3.4	<i>Ae. japonicus</i>	41	2.8
<i>Ae. japonicus</i>	47	2.7	<i>Culex spp.</i>	28	2.7	<i>Cx. pipiens</i>	18	1.2
<i>Cx. pipiens</i>	36	2.0	<i>Ae. japonicus</i>	27	2.6	<i>Aedes spp.</i>	17	1.2
<i>Ps. ferox</i>	7	0.40	<i>Or. signifera</i>	4	0.38	<i>Ur. sapphirina</i>	2	0.14
<i>Ur. sapphirina</i>	5	0.28	<i>Ps. ferox</i>	3	0.29	<i>An. quadrimaculatus</i>	2	0.14
<i>Cx. terriatans</i>	5	0.28	<i>Cx. restuans</i>	2	0.19	<i>Ae. trivitattus</i>	2	0.14
<i>Anopheles spp.</i>	3	0.17	<i>Anopheles spp.</i>	1	0.10	<i>Anopheles spp.</i>	1	0.07
<i>Mansonia spp.</i>	3	0.17	<i>Ae. trivitattus</i>	1	0.10	<i>Ps. ferox</i>	1	0.07
<i>Cu. inoranta</i>	3	0.17	<i>Cx. tarsalis</i>	1	0.10	<i>Or. signifera</i>	1	0.07
<i>Cx. restuans</i>	3	0.17				<i>Psorophora spp.</i>	1	0.07
<i>An. perplexans</i>	3	0.17						
<i>Ae. atlanticus</i>	3	0.17						
<i>Cx. nigripalpus</i>	2	0.11						
<i>Cx. erraticus</i>	2	0.11						
<i>Ae. dupreei</i>	2	0.11						
<i>An. quadrimaculatus</i>	1	0.06						
<i>Ps. ciliata</i>	1	0.06						
<i>Ae. trivitattus</i>	1	0.06						

Table 13 *Continued*. Collected female mosquito totals and percentage yield by year, by CO₂-baited CDC trapping.

<i>Ps. cyanesans</i>	1	0.06	
<i>Ae. sollicitans</i>	1	0.06	
Total mosquitoes – 1765			Total mosquitoes – 1045
			Total mosquitoes – 1460

Table 14. Collected female mosquito totals and percentage yield, by a series of four separate human-landing catches, 2004.

Species	No. of mosquitoes	Percentage
<i>Ae. albopictus</i>	363	69.0
<i>Ae. triseriatus</i>	91	17.0
<i>Ae. trivitatus</i>	30	5.7
<i>Ps. ferox</i>	14	2.7
<i>An. punctipennis</i>	6	1.1
<i>Ae. sticticus</i>	6	1.1
<i>Aedes spp.</i>	4	0.76
<i>Ae. atropalpus</i>	3	0.57
<i>Anopheles spp.</i>	2	0.38
<i>Ae. japonicus</i>	1	0.19
<i>An. perplexans</i>	1	0.19
<i>Ae. canadensis</i>	1	0.19
Total mosquitoes 528		

Table 15. The number and percentage of *Aedes albopictus* and *Aedes triseriatus* collected by time period, by a series of four separate human-landing catches, 2004.

Time period	<i>Aedes albopictus</i> Total (Percentage)	<i>Aedes triseriatus</i> Total (Percentage)	Total
6:00 – 8:00	8 (34.8)	15 (65.2)	23
8:00 – 10:00	19 (90.5)	2 (9.5)	21
10:00 – 12:00	88 (76.5)	27 (23.5)	115
12:00 – 14:00	29 (69)	13 (31)	42
14:00 – 16:00	37 (90.2)	4 (9.8)	41
16:00 – 18:00	75 (88.2)	10 (11.8)	85
18:00 – 20:00	99 (87.6)	14 (12.4)	113
20:00 – 22:00	8 (57.1)	6 (42.8)	14
Total	363 (80)	91 (20)	454

Aedes albopictus collected exhibited two clear bimodal landing peaks between 10:00-12:00 and 16:00-18:00, while *Ae. triseriatus* collected exhibited their largest peak between 10:00-12:00 and a smaller peak between 16:00-20:00 (Figure 12).

The catch per hour for the total number of female mosquitoes collected in 2004, was 0.51 mosquitoes collected per hour for CO₂-baited CDC trapping, and 8.8 mosquitoes collected per hour by human-landing catches. The catch per hour for both *Ae. triseriatus* and *Ae. albopictus* females collected in 2004, was 0.36 and 7.6 per hour by CO₂-baited CDC trapping and human-landing catches respectively.

Statistical analyses. Species, vegetation, and cumulative precipitation were significantly ($P < 0.05$) associated with the number of mosquitoes collected by CO₂-baited CDC trapping (Table 16). These variables could explain 12.12% of the variation in the total number of mosquitoes collected. Species (*Ae. albopictus*), vegetation type (residential), and the cumulative precipitation for the four weeks prior to collection were all found to be significant predictors of total number of mosquitoes collected.

Discussion

Aedes albopictus was more abundant than *Ae. triseriatus* collected by CO₂-baited CDC trapping and by human-landing catches at all sites, vegetation types, at probable and confirmed case sites, as well as sites with no reporting of LACV infections. CO₂-baited CDC trapping in 1998 at two former cases sites of LACV infections demonstrated similar findings [35], and again in 2000 [16]. These results indicate that *Ae. albopictus* is the most abundant diurnal mosquito species collected in eastern Tennessee.

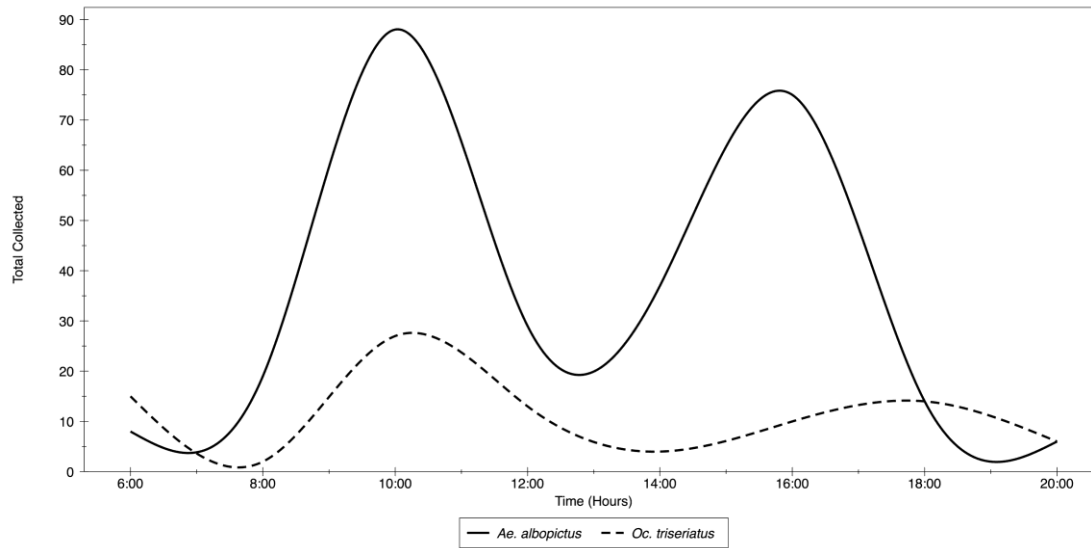


Figure 12. The total number of *Aedes albopictus* and *Aedes* collected by series of human-landing catches* by collection time.

*Mosquito collection totals came from the three separate human-landing catches where *Aedes albopictus* and *Aedes triseriatus* were collected.

Table 16. Final model of the predictors of the number of the adult female host-seeking mosquitoes collected by CO₂-baited CDC trapping in eastern Tennessee, 2004 to 2006.

Parameter	Estimate	<i>p</i> -value	95% Confidence Interval
Species (<i>Aedes albopictus</i> versus <i>Aedes triseriatus</i>)	-3.216	0.000	-3.905, -2.527
Vegetation (Yellow-poplar white-oak northern red-oak versus northern red-oak)	0.668	0.149	-0.239, 1.576
Vegetation (Residential versus northern red-oak)	3.181	0.000	2.117, 4.246
Cumulative precipitation four weeks prior to collection	0.072	0.026	0.009, 0.136

Aedes albopictus adults occurred in both space and time with serologically confirmed cases of LACV infections, as seen in previous studies results support previous findings that *Ae. albopictus* has become more abundant in peridomestic sites than *Ae. triseriatus* [16,35]. The presence of high numbers *Ae. albopictus* at case sites of LACV infections increase the likelihood that this species is playing a role in the transmission and /or maintenance of LACV in this region.

It has been suggested that adult *Ae. triseriatus* may be under-sampled by light trapping and that the use of CO₂-baited traps, battery powered aspiration, and/or human biting collections are better collection methods [122,151,177]. Previous work in the eastern Tennessee has demonstrated that more *Ae. albopictus* are collected than *Ae. triseriatus* using battery powered aspiration [38]. Our data suggest that *Ae. triseriatus* is not under-sampled by CO₂-baited CDC trapping or by human-landing catches (Table 2, 4) in peridomestic sites. It is possible that the differences in the overall abundance of *Ae. triseriatus* and *Ae. albopictus* collected by these methods could be due to the different vegetation preferences of these species.

In 2002, the first blinded cohort study investigating the clinical symptoms and environmental and entomological risk factors of LACV transmission in eastern Tennessee was undertaken [16]. Significant factors found to be associated with LACV transmission were the number of hours per day spent outdoors (5.9 for LACV infection cases vs. 4.0 for non-cases, $p < 0.05$), living in a residence with one or more tree holes within a 100 m radius from the dwelling (relative risk = 3.96, $p < 0.05$), and the burden of *Ae. albopictus* collected at sites (three times greater at cases than non-cases, $p = 0.013$). These data suggest that *Ae. albopictus* could be was playing a greater role in LACV

transmission in eastern Tennessee than elsewhere, e.g. Wisconsin. No statistical difference was found in the burden of *Ae. triseriatus* around residences of LACV infection cases and non-cases. *Aedes albopictus* was more abundant at all vegetation types. This was most apparent at residential sites with three times as many *Ae. albopictus* collected by CO₂-baited CDC trapping as *Ae. triseriatus*. This is likely due to the ability of *Ae. albopictus* to colonize a wide range of environments including residential/urban [91], while *Ae. triseriatus* has traditionally been associated with forested areas [178]. The vegetation preferences of these two species, may also explain why LACV isolations are much lower for *Ae. albopictus* than *Ae. triseriatus* [17].

Aedes triseriatus is known to feed on a variety mammals including deer, squirrels, chipmunks and humans [86,114], while *Ae. albopictus* is considered a catholic feeder and is known to feed on a diverse range of mammalian, avian, and reptilian species [93,114,115]. The differences in feeding behaviors between these two species coupled with differences in vegetation/habitat preferences would make it more likely that a higher proportion of *Ae. triseriatus* would come in contact and to feed on the primary amplification hosts, the eastern chipmunk, *Tamias striatus*, the gray squirrel, *Sciurus carolinensis*, and the fox squirrel *Sciurus niger* [4,25,27,28]. *Aedes triseriatus* likelihood of involvement in horizontal transmission cycle, coupled with its higher midgut infection and dissemination rates of LACV through oral infection [88] make it the likely primary vector of LACV in this region. It is probable that *Ae. albopictus* serves as an accessory vector, though its high abundance and aggressive biting in this region may overcome its shortcomings in its potential to acquire, develop, and transmit LACV.

Increased cumulative precipitation for the four weeks preceding collection was found to have a significant effect on the number of *Ae. albopictus* collected (Figure 9), and may allow *Ae. albopictus* immature stages to out compete *Ae. triseriatus* under certain conditions [179,180]. The presence of stable and/or increasing levels of water in containers and tree holes would also reduce the susceptibility of *Ae. albopictus* eggs to desiccation in drier environments [181,182] and would increase the fitness of *Ae. albopictus* to compete with *Ae. triseriatus* during the larval stage due to a reduction of environmental stress. Residential areas in this region typically have high numbers of artificial containers [35], thus providing increased larval habitat for vector species. Stable and/or increasing water levels coupled with higher temperatures during the summer months would also allow for *Ae. albopictus* to develop at higher rates [183] and more rapidly than *Ae. triseriatus* [179]. Thus, the increased levels of precipitation during larval development would appear likely to have had a positive effect on the development of *Ae. albopictus* relative to *Ae. triseriatus*.

In this study, *Ae. triseriatus* was most frequently captured between 10:00-12:00 with a smaller peak between 16:00-20:00 (Figure 12). These results differed from studies in Wisconsin, where [178] found the greatest biting activity was in the afternoon prior to 18:00 and studies by [184] and [185] found that biting steadily increased from morning until mid-afternoon, though catches made by [185] in August, exhibited a similar bimodal peak in captures as our study reports. These variations could be due the differences in collection methods, replications, climate, terrain, vector competition, and vegetation. Our results are in agreement with Scholl et al. [184] in that the highest catches were made

during periods of the highest humidity (Figure 10). It is likely that *Ae. triseriatus* bites throughout the day, with biting decreasing during the crepuscular period.

Aedes albopictus was captured in two clear bimodal landing peaks; between 10:00-12:00 and 16:00-18:00 (Figure 12). These findings are in agreement with previous studies in east Asia that found two peaks of biting activity in both wooded and open areas [186,187]. Our results differed from these studies only in that the first peak of activity was the highest. The presence of landing (probing/biting) *Ae. albopictus* at confirmed case sites of LACV infections further increases the chances that this species may be transmitting LACV to humans in this region.

Our results demonstrate that the use of human-landing catches to collect these two vector species are more productive than by collections made by CO₂-baited CDC trapping in this region. The three human-landing catches where *Ae. triseriatus* and *Ae. albopictus* were captured accounted for 18 and 30 percent respectively of the total number of these two species collected during the entire 2004 trapping season. An additional benefit to the use of human-landing catches where collections are made in tubes is that the tubes provide for better specimen preservation compared with collections made by CO₂-baited CDC trapping.

LACV infections became seasonally epidemic in eastern Tennessee coinciding with the establishment of *Ae. albopictus*, marking a significant increase in cases in eastern Tennessee from the previous 30 years. This increase in cases could be a result of increased surveillance, diagnosis, and reporting for LACV infections following the outbreak in 1997, and/or could be a result of increased expansion of human settlements into virus foci, as well as due to the introduction and establishments of *Ae. albopictus* in

this region. To date LACV has only been isolated from *Ae. albopictus* in eastern Tennessee. Future studies are needed to address the presence of LACV within vector populations. *Aedes albopictus* is currently the most abundant diurnal mosquito collected in eastern Tennessee by human-landing catches and CO₂-baited CDC trapping associated with LACV.

CHAPTER VII

The Use of the Variable Pressure Scanning Electron Microscope for the Examination of Insect Specimens

This chapter is a lightly revised version of a paper by the same name *IN REVIEW* in the journal *Zootaxa* in 2009 by Andrew D. Haddow, Sachin Deo, and David C. Joy

Haddow, A.D. S. Deo, and D.C. Joy. 2009. The use of the variable pressure scanning electron microscope for the examination of insect specimens. *Zootaxa* In Review

My contributions to this paper are 1) substantial contribution to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published.

Abstract

The use of a variable-pressure scanning electron microscope (VPSEM) has many advantages over the conventional high vacuum SEM, which experiences several inherent weaknesses including: specimen preparation time, the inability to view specimens in their natural fully hydrated state, and the requirement of operating at high vacuum. The use of a VPSEM overcomes the weaknesses of the SEM, minimizing shrinkage, mass loss, and eliminates charging in specimens, as well as the need for sample coatings. These advantages are particularly applicable for observing and imaging medically important insect specimens. We explore the advantages of the VPSEM over the traditional SEM and the techniques for observing and the imaging of insect specimens using this microscope. This note should be of interest to a broad audience of entomologists, in addition to researchers in the various fields of life science.

Introduction

The examination of the fine structures of insect specimens often requires the use of microscopy at the micrometer or smaller scale levels. The viewing of ultrastructural detail is greatly aided by the use of a scanning electron microscope (SEM). However the conventional SEM has several inherent weaknesses including specimen preparation time, the inability to view specimens in their natural fully hydrated state, and the requirement of operating at high vacuum. We explore the advantages of the variable-pressure scanning electron microscope (VPSEM) over the traditional SEM and the techniques for The description and identification of the microscopic fine structures of medically

important insects are often not visible using light microscopy. To overcome this limitation the scanning electron microscope (SEM) has been employed for the observation and imaging of such features [188]. The preparation of insect specimens for imaging using the SEM invariably requires the use of complex and time consuming techniques [189], and operation of the microscope under a high vacuum. Here we examine the advantages of, and report techniques for, viewing and imaging specimens using the variable-pressure scanning electron microscope (VPSEM).

Methodology

In the VPSEM the specimen chamber is held at a finite gas pressure, typically in the range 0.01 to 3 Torr (1 to 400 Pa), rather than under the high vacuum (10^{-6} Torr or 10^{-4} Pa) of the conventional SEM. The pressure is controlled by the operator, and if desirable the contents of the specimen chamber can be changed from the default laboratory air to a pure gas such as helium, or to saturated water vapor. The instrument can also be quickly restored to high vacuum operation by a simple adjustment of the gas injector system.

The VPSEM has numerous advantages over the conventional SEM. Specimen preparation time is greatly reduced as non-conductive specimens do not have to be coated with a thin metallic film and dried prior to imaging. Such coatings are undesirable because they may obscure valuable surface detail, and the process of coating may change the inherent properties of the specimen being viewed. Specimens, including wet or even fully hydrated examples, can also be viewed in their natural state using the VPSEM, which is not possible using the traditional SEM. The use of air or water vapor in the

VPSEM negates the need to employ critical point drying to prepare samples [189], as biological specimens are not prone to collapse and distortion when their water content is maintained and stabilized. The VPSEM operates at higher pressures than the standard high vacuum SEM, and this has the effect of scattering some of the electrons focused on to the specimen. The result of this scattering is a small reduction in the signal level and a corresponding increase in noise, but resolutions of 2 to 4 nm - fully comparable to those obtained in a normal SEM - are still possible when using the VPSEM [190]. The magnitude of the scattering is controlled by using the lowest pressure that will maintain the stability and viability of the specimen, and reducing the working distance (i.e. the distance between the top of the specimen and the bottom of the objective lens of the SEM) to a few millimeters as compared to 10-15mm in a regular SEM.

The following protocol for observing and imaging insect specimens was originally developed for mosquito eggs [191], though these same operational parameters could be applied to any insect specimens made up of soft tissue and containing water. The starting conditions for general observations using the VPSEM for insect specimens are as follows:

- 1) The Beam Energy should be set between 20 to 30keV - lower energies may produce useful images but could result in more noise in the image.
- 2) The beam current should be set to a value of 100pA or higher. The scan rate is most conveniently chosen to be a visual scan rate (i.e. a frame rate of approximately one second) rather than television speed, which may not work

well. If there is any subsequent evidence of damage or mass loss (“radiolysis”) the beam current should be reduced to the lowest value consistent with obtaining an acceptable image.

- 3) The working distance should be set to be no greater than 8mm.
- 4) On most VPSEMs the default imaging mode will use the backscatter detector although a dedicated Environmental SE detector (ESED) is provided on some instruments. The backscatter detector will help to minimize any effects of beam induced charging on the specimen and will provide high contrast detail from regions of the sample differing in chemical composition.
- 5) The gas environment within the chamber can use either laboratory air or, preferably, water vapor. An initial gas pressure of 30Pa (0.25 Torr) is usually suitable, though pressure may need to be increased if there is evidence of charging.

In the event that the sample needed to be kept fully hydrated as in the case of a specimen made up of soft tissue (eg. mosquito larva), then the samples temperature should be lowered to -10°C or below and/or the pressure should be increased to 50-100Pa. These changes will help keep the specimen on the boundary between the liquid and vapor states in the water phase diagram. The lower the temperature the lower the chamber pressure required to keep the sample in a stable hydrated state. The use of

saturated water vapor is generally preferable to normal laboratory air, because it helps minimize charging and distortions of the structure as a result of drying. However providing a reliable source of pure, filtered, water vapor is not easy if extended operation is required.

In the intention is to cool the specimen for observation the following procedure should be employed to load the sample into the VPSEM without allowing it to dry and shrink:

- 1) Load the specimen on the SEM sample stage.
- 2) Cool the specimen to the desired operating temperature e.g. -10C while leaving the sample exposed to the ambient environment.
- 3) With the specimen maintained at its lowest temperature load the specimen into the VPSEM sample chamber and pump the sample to 100Pa or the desired operating pressure.
- 4) Adjust the temperature and pressure to achieve the desired imaging condition.
- 5) To remove the sample from the chamber without causing damage or shrinkage, bring the specimen chamber up to atmosphere pressure while holding the sample at the lowest temperature achieved, then turn off the cooling stage and allow the specimen to warm up in the ambient environment. This sequence of steps will

prevent the specimen from being vacuum dried during pump down.

Summary

In summary, the use of a VPSEM provides many advantages when compared to a conventional high vacuum SEM. These include minimizing shrinkage and mass loss and eliminating charging and the need for sample coatings. These advantages are particularly applicable for observing and imaging insect specimens.

CHAPTER VIII

Description of the Egg of Aedes japonicus japonicus (Diptera: Culicidae) using Variable Pressure Scanning Electron Microscopy

This chapter is a lightly revised version of a paper by the name of “Description of the egg of *Ochlerotatus japonicus japonicus* (Diptera: Culicidae) using variable pressure scanning electron microscopy” published in the *Journal of Medical Entomology* in 2009 by Andrew D. Haddow, John K. Moulton, Reid R. Gerhardt, Linda J. McCuiston, Carl. J. Jones:

Haddow, A.D. J.K. Moulton, R.R. Gerhardt, L.J. McCuiston, and C.J. Jones. 2009. Description of the egg of *Ochlerotatus japonicus japonicus* (Diptera: Culicidae) using variable pressure scanning electron microscopy. *Journal of Medical Entomology* 46: 9-14.

*The genus *Aedes* has been substituted for *Ochlerotatus* in this Chapter for continuity within the dissertation.

My contributions to this paper are 1) substantial contribution to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published.

Abstract

The egg of *Aedes japonicus japonicus* (Theobald) is described with the aid of variable pressure scanning electron micrographs. The egg is black, cigar shaped, and tapers ventrally. The length is approximately 591 μm and the width is approximately 172 μm . The outer chorionic cells are irregular in shape, either hexagonal or pentagonal, and decrease in size towards the anterior and posterior poles. Ventral tubercles typically range from three to six and contact the chorionic reticulum. Dorsal tubercles contain two large tubercles with small oval-shaped tubercles grouped around them. A large thread-shaped tubercle extends from this grouping either as a single tubercle or as a series of connected tubercles. The micropylar collar is low and discontinuous and is seldom complete. This description will aid researchers in the identification of this invasive vector species.

Introduction

The previous range of *Aedes (Finlaya) japonicus japonicus* (Theobald) included Korea and Palearctic Japan [192]; it had also been collected in the former USSR [193]. Since its introduction into the United States in 1999 [95,96] it has spread rapidly and poses a significant threat to the maintenance and transmission of arboviruses. *Aedes japonicus* has been shown to be a competent laboratory vector of La Crosse virus [24], Eastern Equine encephalitis virus [194], St. Louis encephalitis [195], West Nile virus [196], and Japanese encephalitis virus [197]. Recently, this vector has been found at

several case sites of La Crosse encephalitis in eastern Tennessee (A.D. Haddow unpublished).

Herein we describe the egg of *Ae. japonicus* with the aid of a variable pressure scanning electron microscope (VP-SEM). This is the first use of a VP-SEM to describe a culicid egg. There are two partial descriptions of the egg of *Ae. japonicus*, both using traditional low-power SEMs. The first shows the ventral (upper) chorionic surface and cells of a deformed egg [198], and the second shows the ventral (upper) chorionic cells [199]. We present this new data to assist field biologists in the identification of predominantly collected tree-hole species/container species, and to provide more detailed information on characters of Aedini eggs. This brings the number of more complete descriptions and micrographs of the micropyle and associated structures of the subgenus *Finlaya* to three.

Methodology

Embryonated eggs were obtained from a laboratory colony at Rutgers University, New Jersey. The colony was derived from field-collected individuals from Ocean and Somerset Counties, NJ, collected from 2000 to 2001. Blood-fed mosquitoes were provided moist seed germination paper upon which to oviposit. Eggs originated from five different females. Three eggs were randomly selected for analysis from each female. Microscopy was conducted using a Hitachi S-4300 SE/N VP-SEM (Hitachi High Technologies America, Inc). VP-SEM is more versatile than traditional SEM due to a number of factors [200], most notably the ability to view specimens in their natural state, leading to a reduction in specimen preparation cost and time. Measurements were made

using Image J (National Institutes of Health, USA). Terminology follows that of Harbach and Knight [201], and Linely's [202] definition of "outer chorionic cell" and "cell field". Previous work used the mean length and width of the outer chorionic cells, but this procedure does not lend itself well to replication between researchers. To enhance repeatability, the area of the cells was determined. Descriptive statistical analyses were performed using STATA 10.0 (STATA Corp. College Station, TX).

Results

General Features. The egg is black and cigar-shaped, tapers ventrally, and has a conspicuous micropylar collar (Figure 13). The mean length ($\pm$ SE) was $591 \pm 7.6 \mu\text{m}$; mean width was $172.3 \pm 2.6 \mu\text{m}$; the mean length/width ratio was 3.44 ± 0.06 . The outer chorionic cells are mainly hexagonal, though some are pentagonal. Individual cell areas range from 177 to $355 \mu\text{m}^2$. Dehiscence occurs transversely approximately $\frac{1}{4}$ from the anterior pole (Fig 14a, b).

Ventral (Upper) Surface. With the exception of the anterior and posterior poles the chorionic cells are primarily hexagonal and are variable in size (Figure 15a). Within the cell fields tubercles range from three to six in number. These tubercles are typically irregular in size and appear trapezoidal in shape, are flat topped or gently rounded, and are present throughout the cell field (Figure 15b). Spoke-like bridges attach them on at least one side to cell floor and/or to the inner rim of the outer chorionic reticulum. The surface of the cell field is smooth in texture.

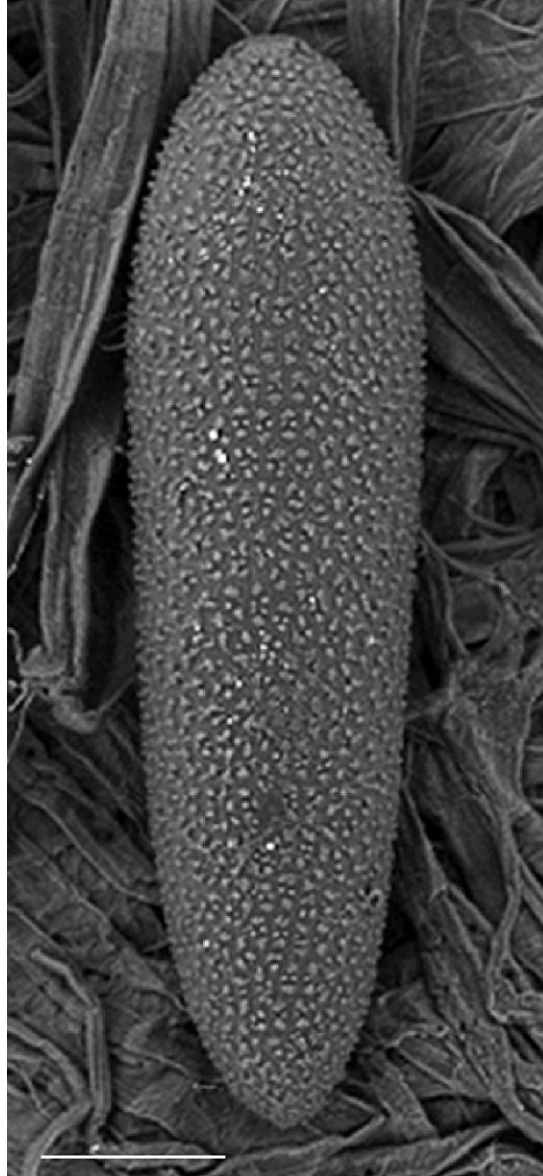


Figure 13. Entire egg. Scale = 100 μm .

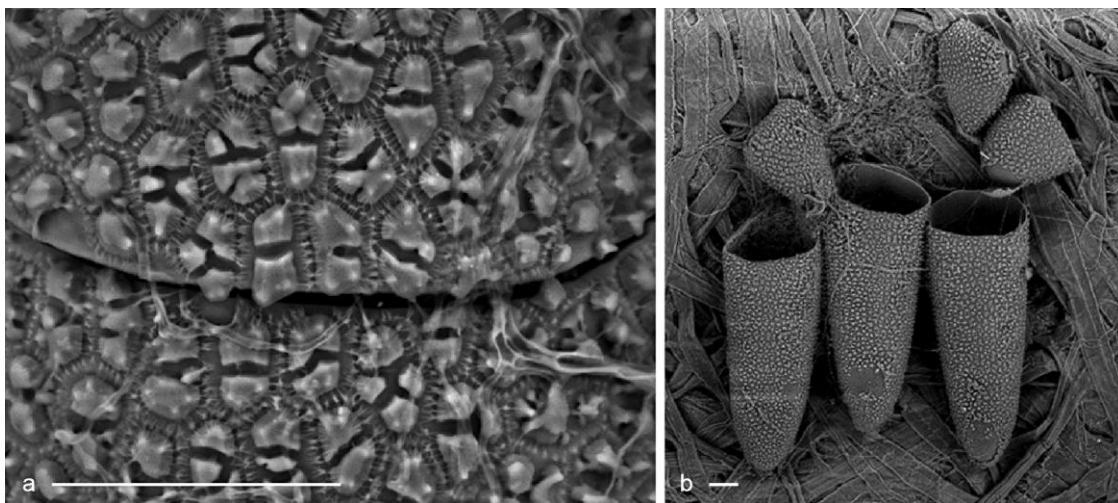


Figure 14. (a) Transverse dehiscence, ventral (upper) surface, approximately $\frac{1}{4}$ from the anterior pole. (b) Hatched eggs with associated egg caps. Scale = 50 μm .

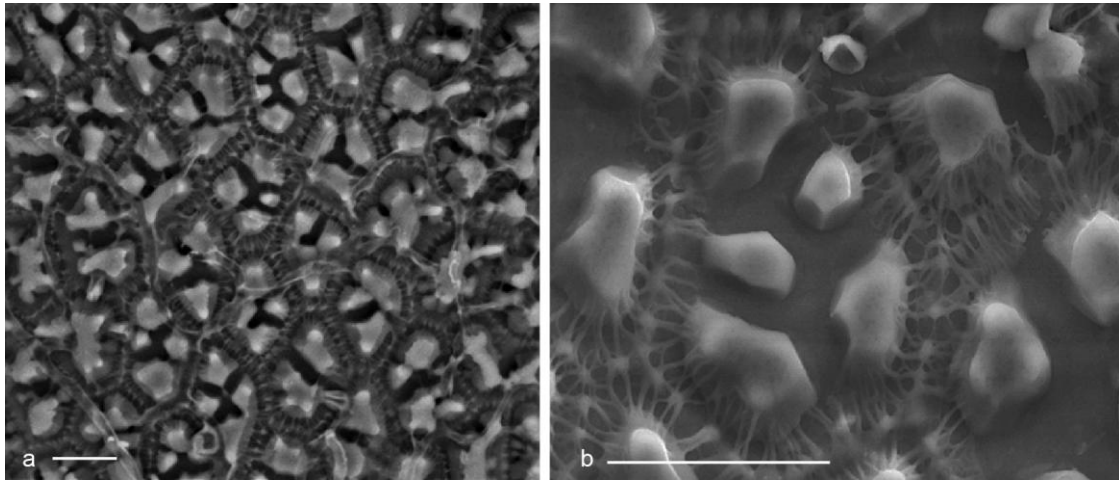


Figure 15. (a) Ventral (upper) surface, typical outer chorionic cells, midway along the length of the egg. (b) Detail of the ventral chorionic cell structure showing five raised tubercles connected to inner rim of the outer chorionic reticulum by spoke-like bridges. Scale = 10 μm .

Anterior Pole and Micropyle. The outer chorionic cells diminish in size approaching the anterior pole (Figure 16a). Tubercles decrease in number to two or three within the cell eventually fusing to each other and the reticulum walls (Figure 16b and c). The micropylar collar is low and discontinuous, with three to four narrow notches present, and is seldom complete (Figure 16d). The micropylar collar width is highly variable, mean width at the narrowest point ($\pm$ SE) was $3.77 \pm 0.15 \mu\text{m}$, mean width at the widest point ($\pm$ SE) was $7.27 \pm 0.27 \mu\text{m}$; micropylar collar mean diameter ($\pm$ SE) was $31.2 \pm 0.98 \mu\text{m}$, the internal diameter ($\pm$ SE) was $17.8 \pm 0.47 \mu\text{m}$; micropylar collar mean depth ($\pm$ SE) was $3.18 \pm 0.19 \mu\text{m}$. The micropylar disk is not clearly defined, is slightly domed, mean diameter of ($\pm$ SE) $11.5 \pm 0.38 \mu\text{m}$. The micropyle itself has a mean diameter ($\pm$ SE) of $1.69 \pm 0.04 \mu\text{m}$.

Posterior Pole. The outer chorionic cells diminish in size approaching the posterior pole (Figure 17a). Tubercles may fuse to each other and the reticulum walls.

Ventral-Dorsal Transition Areas and Dorsal (Lower) Surface. The transitional zone in *Ae. japonicus* is narrow, and occurs rapidly. The dorsal (lower) surface outer chorionic cells become extremely irregular in shape. Each cell contains two large tubercles with small oval shaped tubercles grouped around them; a large thread shaped tubercle extends from this grouping either as a single tubercle or as a series of connected tubercles (Fig 17b).

Discussion

Partial descriptions of *Finlaya* species eggs exist for *Aedes togoi* (Theobald), *Ae. melanopterus* (Giles), *Ae. albopictus* (Theobald), *Ae. formosensis* (Yamada), and *Ae.*

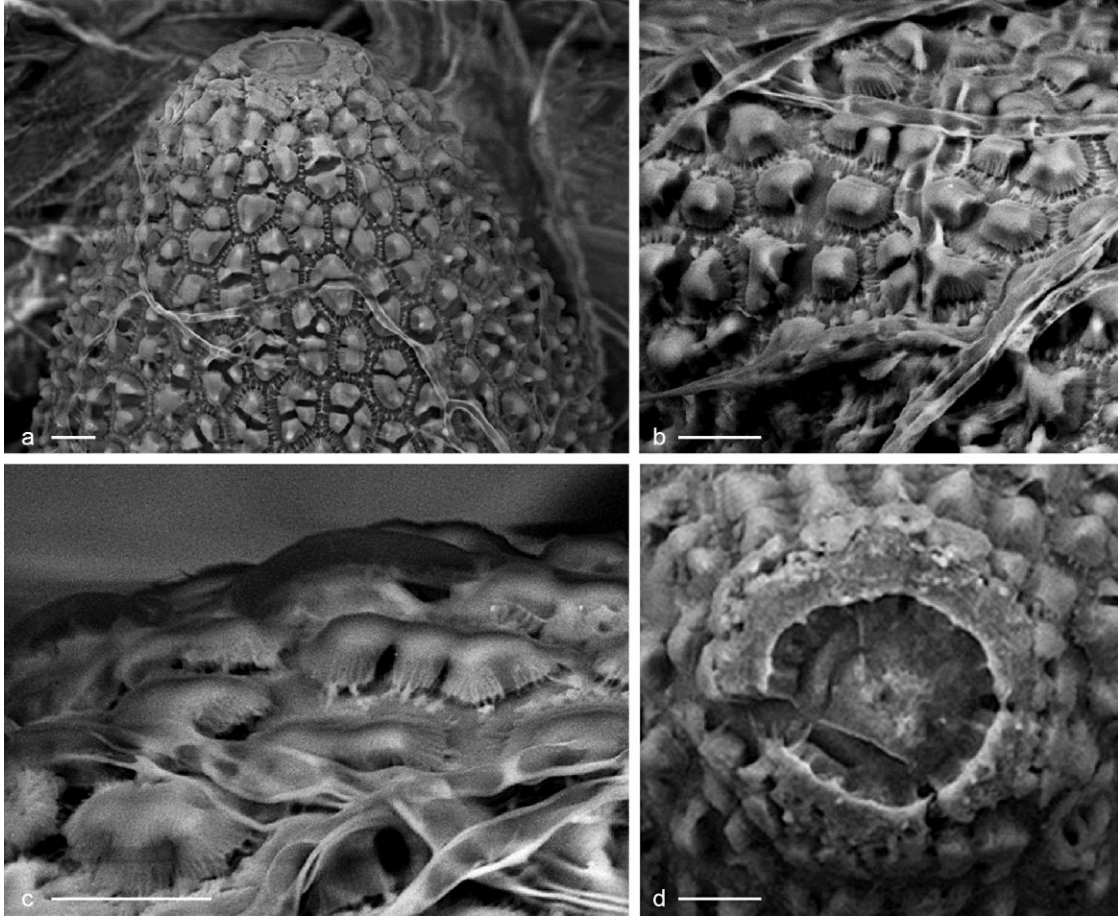


Figure 16. (a) Anterior pole, ventral surface. (b) Outer chorionic cells, ventral surface, approaching the anterior pole. (c) Anterior pole, ventral surface, outer chorionic cell detail. (d) Top view, anterior pole and detail of the micropylar apparatus. Scale = 10 μm .

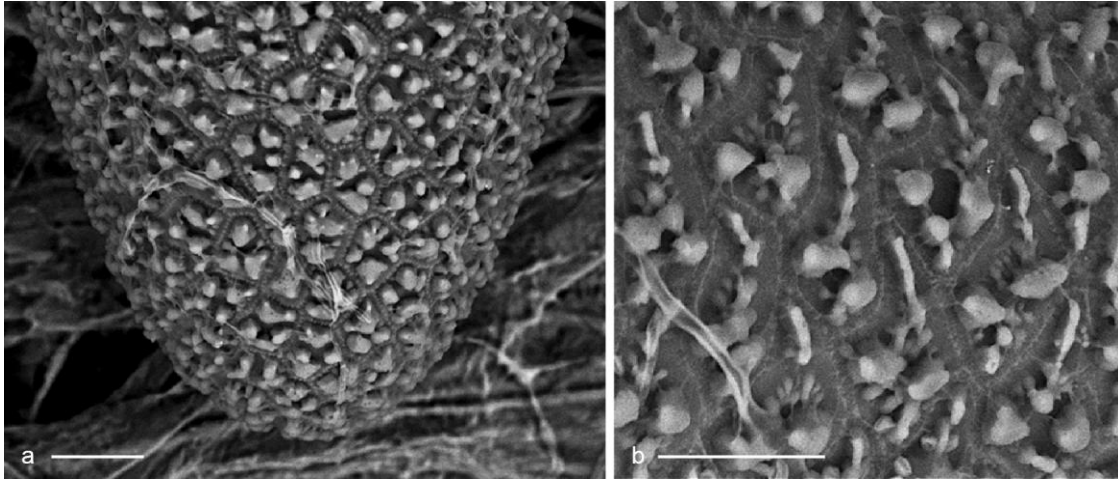


Figure 17. (a) Posterior pole, ventral (upper) surface. (b) Detail of the outer chorionic cells, dorsal (lower) surface. Scale = 20 μm .

japonicus [198,203]. Two more complete descriptions exist for the eggs of *Ae. alboannulatus* (Macquart) and *Ae. rubrithorax* (Macquart) [204]. The characteristics listed in Table 17 form a basis of dimensions that could be useful for the separation of these species. When compared to the descriptions of *Ae. alboannulatus* and *Ae. rubrithorax* [204] the *Ae. japonicus* specimens examined did not exhibit the mid-ventral strip of cells along the dorsal-ventral transition zone. Of the seven *Finlaya* species for which partial and/or complete descriptions exist there are noticeable differences in the fine structure of the eggs [198,203,204]. Further work within this subgenus is needed to determine further differentiating characteristics.

Aedes japonicus has been shown to be a competent laboratory vector of La Crosse virus [24], though its role in the natural maintenance and transmission of the virus has yet to be determined. La Crosse virus was first isolated from the brain of a deceased pediatric patient who died of the virus in 1960 [1]. Since that time confirmed cases of LAC encephalitis have been reported to the Centers for Disease Control in 29 of the lower 48 states [9]. The primary vector in the upper Midwestern United States is *Aedes triseriatus* (Say) [2,20,94]. La Crosse virus has been isolated from *Aedes albopictus* (Skuse) in eastern Tennessee, and evidence suggests that this species may be involved in the maintenance and transmission of the virus in the region [16,17]. *Aedes japonicus* was collected in eastern Tennessee in 2003, and has since become established [99]. All three species' eggs have been collected together in tree-holes, ovitrap cups, and EPS float traps within their range, and therefore differentiating characteristics would prove useful in identifying these species (A.D. Haddow unpublished) (Table 18). The eggs of *Ae. japonicus* and *Ae. triseriatus* are similar in superficial appearance, while the egg of *Ae.*

Table 17. Dimensions of the described eggs of *Aedes (Finlaya)* species.

Species	Length (μm)		Width (μm)		Length/Width (μm)	
	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range
<i>Ae. alboannulatus</i> ^a	700.5 ± 5.9	669.6 – 762.0	187.8 ± 1.9	177.2 – 203.8	3.74 ± 0.05	3.46 – 4.05
<i>Ae. albolateralis</i> ^b	495 ± 2	470 – 510	151 ± 3	120 – 170	NA	
<i>Ae. formosensis</i> ^b	611 ± 4	550 – 650	167 ± 3	120 – 180	NA	
<i>Ae. melanopterus</i> ^b	572 ± 3	550 – 590	169 ± 5	130 – 200	NA	
<i>Ae. rubrithorax</i> ^a	682.3 ± 6.1	632.9 – 727.8	179.6 ± 1.9	165.8 – 192.4	3.81 ± 0.06	3.49 – 4.37
<i>Ae. togoi</i> ^b	516 ± 6	450 – 560	159 ± 4	140 – 180	NA	

^a [204]

^b [203]

Table 18. Differentiating characteristics useful for identifying the tree-hole/container inhabiting mosquitoes involved in the transmission and/or maintenance of La Crosse virus in the United States.

	<i>Aedes japonicus</i>	<i>Aedes triseriatus</i> ^a	<i>Aedes albopictus</i> ^b
Total length range (μm)	545.3 – 636.7	NA	558.8 – 629.4
Mean length ± SE (μm)	591.0 ± 7.6	680.8 ± 9.6	609.8 ± 5.9
Total width range (μm)	161.7 – 186.7	NA	170.6 – 211.8
Mean width ± SE (μm)	172.3 ± 2.6	201.6 ± 2.0	192.9 ± 2.4
Total L/W ratio range (μm)	3.13 – 3.85	NA	1.97 – 3.42
Mean L/W ratio ± SE (μm)	3.44 ± 0.06	3.15 ± 0.05	3.28 ± 0.04
Micropylar collar range (μm)	25 – 38	36.0 (range not given)	45 – 50
Width of micropylar disk range (μm)	Not clearly defined, slightly domed, 9.04 – 15.3	Raised, 18.0	Not clearly defined, domed, 18 - 21
Micropylar orifice (μm)	1.7 ± 0.04 (1.36 – 1.93)	2.0	3.3
Outer chorionic cell shape	Primarily hexagonal, some pentagonal	Irregularly hexagonal, some pentagonal	Hexagonal, occasionally pentagonal
Egg color	Matte black	Dull black	Shiny jet black

^a[205]

^b[202]

albopictus is noticeably different. With regard to size, *Ae. triseriatus* is the largest of the three species and likely could be differentiated from *Ae. japonicus* by length. In terms of fine structure the eggs of *Aedes albopictus*, *Ae. triseriatus* and *Ae. japonicus* differ considerably and can be distinguished when examined under a stereomicroscope. The egg of *Ae. albopictus* is shiny black in color, cigar shaped, tapering anteriorly and posteriorly, chorionic cells are regular in shape and contain one large rounded tubercle with small tubercles present on the inner walls of the outer chorionic reticulum [202]. The egg of *Ae. triseriatus* is dull black in color, cigar shaped, the chorionic cells are irregular in shape, and one large tubercle is usually present touching the outer chorionic reticulum [205]. The egg of *Ae japonicus*, is matte black, cigar shaped, tapering ventrally, the chorionic cells are irregular in shape; tubercles usually range from three to six and contact the chorionic reticulum.

SECTION VI: DISCUSSION

CHAPTER IX

Discussion

Significance

This project set out to determine the current spatial and temporal patterns of disease risk for LACV at the national level, and then within Tennessee and West Virginia, and to determine the current vector ecosystem dynamics in eastern Tennessee. The results of studies have led to the creation of a national risk map, in addition to risk maps for West Virginia and eastern Tennessee, provided a national epidemiological picture of LACV infections, led to a further differentiation of the clinical presentation of LACV infections, provided additional information on the seasonal abundance, habitat preferences, and host-seeking behaviors of vector species in eastern Tennessee, provided further biological knowledge of vector species, and further incriminated *Ae. albopictus* as an accessory vector of LACV in eastern Tennessee. These findings will allow focused national serological studies, form the basis for the development of predictive models of virus transmission, provide a methodology for the use spatial analyses at a national level for other infectious diseases, give specific recommendations for the reporting of arboviral disease cases, and will guide interventions by public health officials.

Chapter III

The findings of a higher incidence risk and case-fatality rate indicate a much higher burden of disease than previously reported, and demonstrate that LACV infections are much more common than previously reported. We have demonstrated the usefulness of spatial statistical techniques to detect hot-spots of infections at a national level, thus

allowing for targeted interventions by public health officials while raising awareness among health care providers of geographic areas at the highest risk of disease.

This study provides the first true risk map of LACV for the United States, and presents insights into the clinical picture of LACV infections. The results will allow focused national serological studies, form the basis for the development of predictive models of virus transmission, provide a methodology for the use spatial analyses at a national level for other infectious diseases, and demonstrate the need for the reporting of arboviral and other disease cases at smaller geographic scales.

Chapter IV

Focal arboviral infections affecting a sub-set of the overall population present an often over-looked set of challenges in the assessment and reporting of risk and the detection of spatial patterns. The objective was to assess the variation of risk when using different at-risk populations and geographic scales for the calculation of incidence risk and the detection of disease clusters. We explored these variations using a pediatric arbovirus, LACV, as our model.

The results of this work indicate the possibility of missing disease clusters resulting from performing incidence risk investigations of focal diseases using inappropriate at-risk populations and/or large geographic scales. Public Health efforts to improve both disease surveillance and health planning would be better improved through the assessment of risk in well-defined at-risk populations using the right geographic scale for a specific analysis. This ensures that public health efforts to control disease occurrence are as efficient as possible.

Chapter V

The results of this study demonstrates that south-central West Virginia remains a focus of LACV transmission and highlights the utility of using the combination of incidence risk and spatial statistics to detect areas of high virus transmission. West Virginia continues to have the highest reported case-fatality rate in the United States. Clusters of high risk were detected in areas where previous work indicated a high presence of *Ae. japonicus* and *Ae. triseriatus*, and low numbers of *Ae. albopictus*. The presence of wooded areas and artificial containers in virus foci remain a risk factor for acquiring LACV. These findings will allow public health officials to target these areas for interventions.

Chapter VI

Aedes albopictus was found to be the most abundant mosquito collected at all sites and vegetation types, by both CO₂-baited CDC trapping and human landing catches. It exhibited two clear peaks of “landing” activity, one in the early morning and one in the late afternoon or early evening. Species (*Ae. albopictus*), vegetation (residential), and the previous cumulative precipitation for the four weeks prior to collection were significantly ($P < 0.05$) associated with the number of mosquitoes collected by CO₂-baited CDC traps. This work further implicates *Aedes albopictus* as an accessory vector of LACV in eastern Tennessee.

Chapter VII

The use of a VPSEM has many advantages over the conventional high vacuum SEM, which experiences several inherent weaknesses. The use of a VPSEM overcomes

the weaknesses of the SEM. These advantages are particularly applicable for observing and imaging medically important insect specimens. This work explored the advantages of the VPSEM over the traditional SEM and the techniques for observing and the imaging of insect specimens using this microscope. This work is relevant to a broad audience of entomologists, in addition to researchers in the various fields of life science.

Chapter VIII

Prior to this work, no complete description of the egg of *Ae. japonicus* existed in the literature, hampering efforts to identify and distinguish eggs collected during ovitrapping from other species. This work will assist field biologists in the identification of the egg of *Ae. japonicus* and other predominantly collected tree-hole species/container species, provide more detailed information on characters of Aedini eggs, while bringing the number of more complete descriptions and micrographs of the micropyle and associated structures of the subgenus *Finlaya* to three.

Future Research

These results of the studies in Chapters III, IV, and V infer the wide variation in risk that occurs due to the focal nature of the virus. Future work is needed to develop predictive models for LACV that incorporate vector competence in different geographic regions, host data, climate data, vegetation data, and case data. Ecological niche modeling of LACV virus in an endemic area e.g. West Virginia, could form the basis of a national model.

It is clear from the results of Chapters III, IV, and V that the vast majority of cases remain undiagnosed and/or underreported, and the results from previous work

elsewhere support this hypothesis, with estimates of asymptomatic infections to clinical infections in pediatric populations ranging from 2:1 to 1500:1 [13,36,124]. There is a current need for serosurveys in LACV endemic areas, to gauge the prevalence of the virus in these populations. Such work could give clues to the virulence of strains circulating in various different geographical regions while allowing guided health intervention efforts to those areas of highest risk.

It is clear from the results in Chapters III, IV, and V as well as previous work elsewhere [2,29,45,46,118,131] that the vast majority of severe LACV infections occur children, but the question still remains why? This could be due to a variety of factors including differences in the pediatric and host immune system, the dose of virus as children would receive a proportionally larger dose than adults by weight, and/or exposure. These hypotheses need to be studied further, and may shed light on virus/host interactions in other systems.

There remain relatively few virus isolates obtained from vector species in the Appalachian region of the United States [9,17,36], and work is needed to confirm the current status of *Ae. triseriatus*, *Ae. albopictus*, and *Ae. japonicus* as vectors of LACV in this region. Chapters III, IV, V, and VI and previous studies in West Virginia [18,19,33], indicate the possibility of *Ae. albopictus* serving as an accessory vector in eastern Tennessee and/or *Ae. japonicus* serving as an accessory vector in West Virginia, but the current status of these vectors remains unknown. Future studies are needed to address these questions.

Recommendations

Currently, state health departments report LACV infections at the county level, but the results of Chapter IV have demonstrated the sub-county level ie the census tract would provide a more accurate picture of disease risk, while reducing the chances of missing disease clusters using Global and Local Morans I statistics. It would therefore be beneficial to for state health departments to begin reporting LACV cases to the CDC at the census tract level.

Traditionally, LACV infections have been reported as LAC encephalitis [5]. Such reporting distorts the true picture clinical presentation of severe LACV infections. LACV infections present as meningioencephalitis, encephalitis, meningitis, or uncomplicated fever, Chapters III and V. The accurate long-term reporting of the clinical presentation will allow for comparisons to be made between age, sex, and geographic region and may provide clues to the virulence of different LACV strains.

The significantly high risk clusters detected in Chapters III, IV, and V should be used by public health officials to guide intervention efforts in those areas of highest risk. Public heath officials should continue to educate the public on possible risk factors for acquiring LACV infections, such as age, time outdoors and the presence of disease foci, Chapters III, IV, V, and VI.

The examination of the fine structures of insect specimens requires the use of microscopy at the micrometer or smaller scale levels. Traditionally, conventional SEM and critical point drying are used to facilitate the viewing of these specimens, but suffer from inherent weaknesses. The use of VPSEM overcomes these weaknesses, and should

be used when possible to view specimens, allowing specimens to be viewed in their natural states while preventing valuable surface detail from being obscured, Chapter VII.

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VITA

Andrew Douglas Haddow was born in Iowa City, Iowa on the 10th of April 1978. He grew up in Springfield, Missouri, the son of Alastair Haddow and of Melissa Ann (née Beach). Alastair is an infectious disease specialist at St. Johns Regional Health Center in Springfield, and Melissa is the executive director of the Community Partnership of the Ozarks in Springfield. He has two younger sisters, Catherine (‘Katie’), who is a Nurse Practitioner at St. Johns Regional Health Center in Springfield, and Lauren who is currently in Graduate School at Missouri State University in Springfield pursuing a Masters of Science in Nursing.

Andrew attended Glendale High School in Springfield where he did honors projects on “the influence of the media on presidential elections” in History and on “ the identification and growth of bacteria in free-standing water in a domestic setting” in Biology. He studied Biology and Environmental Science at Drury University in Springfield where he completed research projects on “the study of resistance patterns of bacteria in a tertiary health care center” in Biology and conducted the first phase I assessment of “the possible cause and the effects of point and non-point source pollution on the Jordan Creek ecosystem” in Environmental Science. He graduated with a Bachelors of Science degree in 2001, with concentrations in Biology and Environmental Science.

Andrew then pursued a Masters Degree in Environmental Science at The Johns Hopkins University, graduating in 2003. It was during this time that he worked in the laboratory of J.P. Dubey at the United States Department of Agriculture on the biology *Toxoplasma gondii* and *Besnoitia* species. After his Masters degree he went to the

London School of Hygiene and Tropical Medicine and the Natural History Museum, completing a short course in Vector Biology and Identification in 2003. Upon his return to the United States he began work on a Doctor of Philosophy degree at the University of Tennessee on the epidemiology of La Crosse virus in Tennessee and West Virginia. Following completion of his PhD he will be taking a position as a postdoctoral fellow at the University of Texas Medical Branch in Galveston, working on the “Mechanisms of Sylvatic Dengue Emergence.”