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A NEW GENETIC VARIANT OF LA CROSSE VIRUS (*BUNYAVIRIDAE*) ISOLATED FROM NEW ENGLAND

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Abstract. La Crosse virus (LACV) is found primarily in the Midwestern and Appalachian regions of the United States where it is a leading cause of mosquito-borne encephalitis in children. To determine whether the distribution of this virus extends further east into New England, we analyzed a bunyavirus that was isolated from a pool of eastern tree-hole mosquitoes, *Ochlerotatus triseriatus* (= *Aedes triseriatus*), collected from Fairfield, Connecticut (CT) in 2005. Nucleotide and encoded amino acid sequences from portions of the S, M, and L segments were more similar to the prototype strain of La Crosse virus than that of closely related snowshoe hare virus. Phylogenetic analysis of sequences from the M segment indicated that the CT isolate represents a distinct lineage of La Crosse virus, diverging earliest from other strains found in southeastern, central, and northeastern United States. Despite low sequence homology with other viral strains, the CT isolate was antigenically similar to the prototype strain of LACV by plaque-reduction neutralization tests with polyclonal and monoclonal antibodies. This represents the first isolation of LACV in New England to our knowledge and suggests long-term independent evolution of the CT isolate.

INTRODUCTION

La Crosse virus (LACV) belongs to the California serogroup of the genus *Orthobunyavirus*, family *Bunyaviridae* and is an important cause of mosquito-borne encephalitis in the United States, accounting for about 100 cases reported annually to the Centers for Disease Control and Prevention (CDC).[1] LAC encephalitis afflicts mainly children (< 15 years old) with the majority of cases reported from Midwestern and Appalachian regions of the United States.[2] Transmission of LACV is associated with hardwood forests that support dense populations of the main vector species, *Ochlerotatus triseriatus*[3,4] (= *Aedes triseriatus*, see Reinert and others[5]). (We have adopted the designation of *Ochlerotatus* to the generic rank on the basis of morphologic characters and recent molecular evidence.[6]) The virus persists by transovarial transmission (TOT) from female mosquitoes to their progeny[7,8] and is amplified in a cycle involving mosquitoes and arboreal rodents (eastern chipmunk and gray squirrel).[9–13]

La Crosse virus has been isolated from 13 states in the eastern United States extending west from Texas to Minnesota and east from New York to Georgia.[4] Nevertheless, clinical cases of California group encephalitis have been reported from residents of 28 eastern states,[1] suggesting a broader distribution of the virus. In northeastern United States, the virus has been found exclusively in New York State,[14] despite surveillance efforts in neighboring New England states, New Jersey, and Pennsylvania. Serological evidence of LACV infection, however, has been detected in white-tailed deer, eastern chipmunks, and gray squirrels from western Massachusetts[15] and in cottontail rabbits from Pennsylvania.[16]

In 2005, we isolated a bunyavirus from *Oc. triseriatus* collected from Fairfield, Connecticut (CT) that was identified as LACV by serological and genetic analysis. It is not clear whether the CT isolate represents a recent introduction of LACV from another locality or constitutes a regionally distinct variant of the virus, suggesting a continued existence of LACV within this area. Accordingly, to evaluate its origins, we compared the CT isolate to 15 other geographic strains of LACV by phylogenetic analysis of M segment sequences.

MATERIALS AND METHODS

Mosquito collections. Mosquitoes were trapped at 91 locations statewide from the beginning of June through the end of October 2005.[17] Each trapping site was visited every 7–10 days and mosquitoes were sampled using a CO_2-baited CDC light trap and a CDC gravid mosquito trap. Adults were transported back to the laboratory alive and sorted by sex, species, and trapping location on chill tables using taxonomic keys of Andreadis and colleagues 2005.[18] Mosquitoes were combined into pools of 50 or less and stored at –80°C until virus testing.

Virus isolation and identification. Mosquito pools were placed in 2-mL microcentrifuge tubes containing a copper BB and homogenized in 1 to 1.5 mL of phosphate buffered saline (PBS) containing 30% heat-inactivated rabbit serum, 0.5% gelatin, and 1X antibiotic/antimycotic using a vibration mill as previously described.[17] Mosquito homogenates were centrifuged at 4°C for 10 minutes at 520g and a 100-uL aliquot of the supernatant was inoculated onto a monolayer of confluent Vero cells growing in minimal essential media, 5% fetal bovine serum, and 1X antibiotics/antimycotics. Cells were maintained at 37°C in 5% CO_2 and examined daily for cytopathic effect (CPE) from day 3 through day 7 post-inoculation. Infected cell supernatants were harvested and stored at –80°C until further testing.

RNA was extracted from viral stocks using the viral RNA Kit (Qiagen, Valencia, CA) and eluted in a final volume of 70 uL. Reverse transcription polymerase chain reaction (RT-PCR) was performed using the Titan One-Tube RT-PCR System (Roche Diagnostics, Indianapolis, IN) and primer pair BUNS+new (TGACCAGTAGTGTACTCCAC) and BUNS–new (CAAGCAGTAGTGTGCTCCAC),[19] which target the terminal-ends of the S segment of the *Orthobunyavirus* genus. For each RT-PCR reaction, 2 uL of extracted RNA was added to Master mix I containing 500 uM ATP, 500 uM GTP, 500 uM CTP, 500 uM TTP, 12.5 uM DTT, 1 uM of each primer in a final volume of 20 uL. This mixture was heated to 85°C for 5 minutes and then quick chilled on ice to denature RNA. Master mix I was added to a second master

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mix containing 5X RT-PCR buffer (10 uL) and Titan enzyme mix (1 uL) for a final volume of 50 uL. Amplification was performed as follows: 1 cycle of 50°C for 30 minutes and 94°C for 2 minutes, 10 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 68°C for 1 minute, followed by 25 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 68°C for 1 minute + 5 seconds per cycle, and 1 cycle of 68°C for 7 minutes. S segment amplification products of approximately 950 bp in size were digested with restriction enzymes EcoRV, Cac81, SwaI, and XhoI in separate reactions to identify bunyaviruses known to occur in CT. Predicted restriction fragment polymorphisms for BUNS+/– new amplification products are as follows: EcoRV cuts only Jamestown Canyon virus (fragment sizes 364 and 627 bp) and Keystone virus (356 and 598 bp); Cac81- Potosi virus (256 and 677 bp) and California encephalitis virus (183 and 795 bp); SwaI- Cache Valley virus (411 and 539 bp), and XhoI- Trivittatus virus (233 and 740 bp). Digestion products were separated on a 2% agarose gel and visualized by staining with ethidium-bromide.

Genetic characterization. The CT isolate (6716-05) was further characterized by nucleotide sequencing of amplification products from genomic segments S, M, and L. In addition, a 1,683 nucleotide portion of the M segment, encoding the envelope glycoprotein G2, nonstructural protein NSm, and a portion of the G1 envelope glycoprotein, was sequenced from 8 other isolates of LACV (Table 1) to include in phylogenetic analyses. PCR primers M14C (CGGAATTCAGTAGTGTACTACC) and M4510R (ATCGCGTAGTAGTGTGCTACC) were used to amplify the entire M segment (~4,500 bp) using conditions described previously with modifications to the thermal cycling conditions as follows: 1 cycle of 45°C for 30 minutes and 94°C for 2 minutes, 10 cycles of 94°C for 15 seconds, 48°C for 30 seconds, and 68°C for 4 minutes, followed by 25 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 68°C for 4 minutes + 5 seconds per cycle, and 1 cycle of 68°C for 7 minutes. A portion of the L segment (~650 bp) was amplified using primers L612C (GTGATTTTACACTGACAGCACC) and L1254R (CTAAGATGAATTGTTGTTCCCATAA) and modifying the cycling conditions described for the M segment by reducing the 68°C extension step of 4 minutes to 1 minute. Amplification products of the appropriate size were purified using the PCR purification kit (Qiagen, Valencia, CA) and commercially sequenced (Keck Center, New Haven, CT). Sequence from one of the isolates, W-17258 (ODH102), was derived from plaque-purified virus stocks because sequence chromatograms from the original stock virus could not be interpreted at some nucleotide positions. Viral plaques were developed on Vero cells as described below and a total of 9 plaque-forming units (PFUs) of varying sizes were individually picked, re-passed in Vero cells, and sequenced, all of which yielded identical, unambiguous sequence.

Overlapping sequence chromatograms were aligned and edited using the ChromasPro editing program (Technelysium Ltd, Tewantin, Australia). Multiple sequence alignments were generated by the ClustalW algorithm using Mega 3.0.[20] Phylogenetic relationships were evaluated by maximum likelihood and maximum parsimony analysis of M segment sequences using PAUP 4.0.[21] Maximum-likelihood searches were performed by the heuristic search method with the general time reversible model using codon position-specific rates. Maximum parsimony trees were estimated by the heuristic search method and treating characters as equally weighted and unordered. Tahyna, Lumbo, California encephalitis, and San Angelo viruses served as outgroup taxa to root phylogenetic trees. Support for individual nodes was evaluated by performing 1,000 bootstrap replicates in maximum parsimony analysis.

Plaque reduction neutralization (PRN) test. Serum dilution PRN tests were performed on Vero cell monolayers according to standard protocols.[22,23] The following immune reagents were used: hyper-immune mouse ascitic fluids raised against LACV, hamster antiserum directed against snowshoe hare virus (SSHV), and a monoclonal antibody (807-18) specific for LACV.[24] Approximately 200 PFUs of virus were added to 2-fold dilutions of antibody and this mixture was incubated at 4°C overnight. Each antibody-virus mixture (100 uL) was inoculated onto confluent Vero cells growing in 6-well plates and virus was absorbed at 37°C for 1 hour with periodic rocking. Cell monolayers were then overlaid with 3 mL of 1%

TABLE 1
Characteristics of LACV isolates analyzed in this study

Isolate	Geographical origin	Year	Source (donor)*	Passage history	Accession numbers	References
Prototype	Dresbach, MN†	1960	Human	SM3, BHK3	U18979	40
A3-4851	Baldwin County, AL	1963	*Ps. howardii* (UTMB)	SM3	DQ426682	This paper
W-17258 (ODH 102)	Defiance County, OH	1965	*Oc. triseriatus* (UTMB)	SM4, V1	DQ426683	This paper
74-32813	Albany County, NY	1974	*Oc. triseriatus*	SM4, BHK4	D10370	41
76-40	Richland County, WI	1976	*Oc. triseriatus*	SM2, BHK1	U70206	42
78V-8853	Rochester, MN	1978	*Oc. triseriatus* (CDC)	V1, SM2	DQ426680	This paper
78V-13193	Cherokee, NC	1978	*Oc. triseriatus* (UTMB)	SM1, V2	DQ426681	This paper
22988-89	De Soto, WI	1978	Human	SM2, BHK2	U18980	40
79-283	Crawford County, WI	1979	*Oc. triseriatus*	SM2, BHK1	U70207	42
81-4	Washington County, WI	1981	*Oc. triseriatus*	SM2, BHK1	U70208	42
88-23128	Tifton, GA	1988	Dog (CDC)	V1, SM1	DQ426684	This paper
R56869	Stone County, MO	1993	Human	V1	U70205	42
WV96-653	Nicholas County, WV	1995	*Oc. triseriatus* (CDC)	V1	DQ426685	This paper
NC97-7306	Swain County, NC	1997	*Oc. triseriatus* (CDC)	V1	DQ426686	This paper
TN00-2266	Anderson County, TN	1999	*Ae. albopictus* (CDC)	V1	DQ426687	This paper
6716-05	Fairfield, CT	2005	*Oc. triseriatus*	V1	DQ426688	This paper

SM, sucking mouse; BHK, baby hamster kidney cells; V, Vero cells.
* Virus isolates donated for this study: Drs. Roger S. Nasci and Barbara W. Johnson of the Centers for Disease Control and Prevention (CDC), Ft. Collins, CO and Dr. Robert B. Tesh of University of Texas Medical Branch (UTMB), Galveston, TX.
† Site of human infection (virus isolated in WI).

Agar in minimal essential medium with 2.5% fetal bovine serum and antibiotics/antimycotics. Monolayers were incubated at 37°C, 5% CO_2 for 4 days and then received a second 2.5- mL overlay containing neutral red stain at a final concentration of 1:5,000. Cells were incubated for an additional day before plaques were counted.

RESULTS

An unknown virus (6716-05) was isolated from a pool of four *Oc. triseriatus* collected in Fairfield, CT on 8/15/05. The virus was screened by RT-PCR using primers BUNS+new/BUNS–new designed to amplify the S-segment of California and Bunyamwera serogroup bunyaviruses. Primers yielded an amplification product of the appropriate size (~950 bp) that could not be cut with any of the diagnostic restriction enzymes (EcoRV, Cac81, SwaI, and XhoI) for identifying Jamestown Canyon, Potosi, Cache Valley, and Trivittatus viruses. The CT isolate was further characterized by nucleotide sequencing of the S-segment amplification product. LACV was the most closely related virus based on comparison of nucleotide and amino acid sequences encoding the nucleocapsid, nonstructural protein NSs, and 3′ nontranslated region (Table 2). Further sequencing of amplification products from M and L segments indicated a closer relationship with the prototype strain of LACV than sister taxa- SHHV; however, some of the genes were only slightly more similar to LACV, including envelope glycoproteins G1 and G2.

To determine whether the CT isolate represented an antigenically distinct bunyavirus, the virus was characterized by PRN tests using polyclonal and monoclonal antibodies (Table 3). PRN titers of the isolate were not significantly different from those of the prototype strain of LACV, as defined by a 4-fold or greater difference in titer, and these viruses could be readily distinguished from SSHV using a LACV monoclonal antibody and SSHV antisera. Mouse ascites fluid raised against LACV was broadly reactive against all of the viruses and only a 2-fold difference in titer was observed between LACV and SSHV.

Our initial analysis indicated that the CT isolate represents a genetic variant of LACV. To evaluate possible origins of this virus, we compared it to other geographic strains of LACV by phylogenetic analysis of M segment sequences. Trees generated by maximum likelihood (Figure 1) and maximum parsimony (data not shown) were essentially identical except the branching order of isolates TN99 and NC78 within lineage 1 could not be fully resolved by parsimony analysis. LACV isolates from the Midwest (OH, MN, MO, WI) and Appalachian regions (NC, TN, WV) formed a genetically distinct clade (mean pairwise distance within group = 3.9%), designated as lineage 1. Substructure within this lineage revealed a homogeneous cluster of variants from Minnesota, western Wisconsin (WI 76, WI 78), and Missouri, and another group from Wisconsin (WI 79, WI 81) and western Ohio that corresponded to types A and B in previous RNA fingerprinting studies.[25,26] Our inclusion of additional isolates from the Appalachian region blurred the distinction between these two genetic groups and therefore, we classified them into a single lineage. Lineage 2 comprised strains from the southeastern (AL, GA) and northeastern (NY) United States (mean pairwise difference within group = 2.9%) that corresponded to type C as previously defined.[25] The CT isolate represented the sole member of lineage 3 that was ancestral to all of the other LACV strains (mean pairwise distance = 14.6%).

TABLE 2
Sequence comparison of the CT isolate (6716-05) with prototype strains of LACV and SSHV

Source of sequence	Gene or region	Type of sequence	No. of sites analyzed	% Sequence identity of CT isolate w/ LACV	% Sequence identity of CT isolate w/ SSHV
S-segment	N	nt	707	95.6	87.9
		aa	235	100	90.6
	NSs	nt	276	98.9	92.8
		aa	92	97.8	84.8
	3′ NTR (partial)	nt	146	88.7	86.6
M-segment	G2	nt	858	88.6	82.2
		aa	286	98.3	97.9
	NSm	nt	522	81.6	79.3
		aa	174	91.4	85.6
	G1 (partial)	nt	264	82.6	76.1
		aa	88	88.6	85.2
L-segment	Pol (partial)	nt	513	85.2	80.9
		aa	171	95.3	90.6

N, nucleocapsid; NSs and NSm, nonstructural proteins; G1 and G2, envelope glycoproteins; NTR, nontranslated region; Pol, RNA polymerase.

TABLE 3
Results of PRN tests using polyclonal and monoclonal antibodies (Mab)

Virus	Reciprocal 90% PRN titer of antisera/Mabs LAC	SSH	LAC Mab
CT isolate (6716-05)	20,480	80	20,480
LAC (prototype)	20,480	80	10,240
SSH (prototype)	10,240	320	< 20

Titers are expressed as the highest serum dilution that neutralized 90% of plaques.

DISCUSSION

We find that the geographic range of LACV extends further east than previously recognized and includes New England. Our isolate from CT proved to be distantly related to other geographic strains of LACV; nevertheless, neutralization titers were not significantly different from the prototype strain of LACV.

By phylogenetic analysis, we were able to distinguish three lineages of LACV circulating in different geographic regions of the United States. The CT isolate was the only member of a newly recognized lineage that apparently diverged earliest from other LACV strains. This supports the hypothesis that LACV has persisted within this region for a long duration of time but has gone undetected. Alternatively, this lineage may have been recently introduced from another geographic locale that was not included in our analysis. Prior RNA fingerprinting studies included an additional 22 strains to those represented here and all were assigned to one of the established genetic types—A, B, or C—which correspond to lineages 1 and 2 in this study.[25,26] Thus we believe that lineage 3 is sufficiently different from the other genotypic groups that its presence would have been apparent by genetic fingerprinting techniques. A final possibility is that lineage 3 was recently introduced from another geographic region outside of the known distribution of LACV. Indeed, the recent description of another virus related to SSHV and LACV from Russia

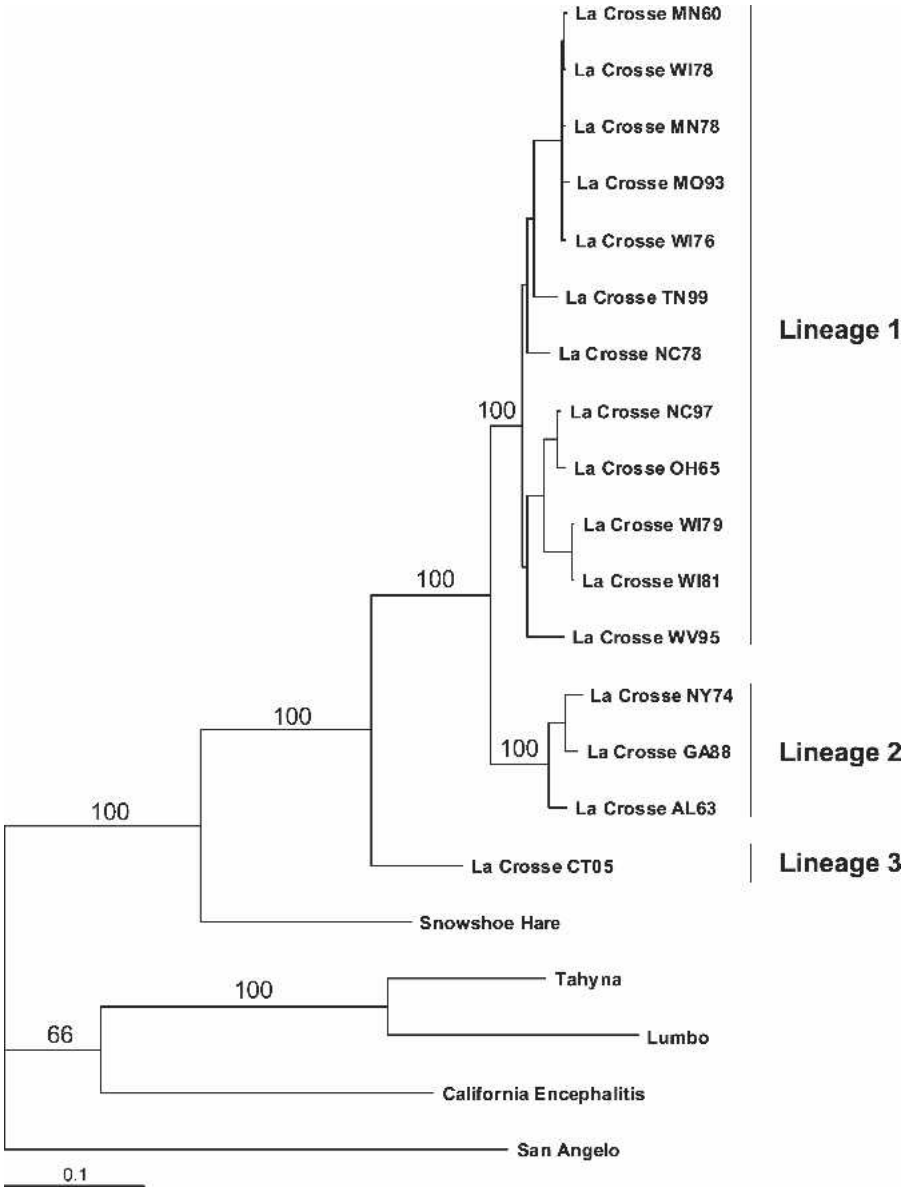

FIGURE 1. Phylogenetic relationships of LACV isolates based on maximum-likelihood analysis of nucleotide sequences from the M segment (1,686 bp). Numbers above branches represent bootstrap support for 1,000 replicates from maximum parsimony analysis. Bootstrap values for nodes within lineages are omitted for clarity. Viruses are designated by the state and year of isolation.

suggests a wider diversity and distribution of these viruses than previously recognized.[27]

Despite long-term independent evolution of the CT isolate, its antigenic features appear to be highly conserved and characteristic of LACV. Neutralization titers of the CT isolate were similar to the prototype strain of LACV yet distinct from closely related SSHV. This relationship was most apparent when using a monoclonal antibody targeting the G1 surface glycoprotein of LACV.[24] These results indicate shared antigenicity with the prototype strain, yet further probing with monoclonal antibodies may reveal neutralization determinants that are specific for the CT isolate. Moreover, given the extent of genetic differentiation between the CT isolate and other geographic strains of LACV, it is possible that these viruses exhibit differences in other phenotypic traits such as viral replication rates, infectivity, host-specificity, and virulence. This possibility merits further investigation because such differences in viral phenotype could be relevant to the epidemiology of LACV within different regions of the United States.

We had previously suspected that LACV may circulate in CT because of its geographic proximity to enzootic sites in New York and because of the similar ecological conditions for maintaining LACV transmission in southern New England. This region is characterized by fragmented woodlands that support *Oc. triseriatus* and the main amplification hosts (eastern chipmunk and gray squirrel). Nevertheless, transmission appears to be less intense within this region than in other parts of eastern US. Of 19,023 *Oc. triseriatus* sampled in CT, only one pool yielded LACV (minimal infection rate- MIR per 1,000 mosquitoes = 0.05), representing our sole isolate of the virus during statewide surveillance from 1997–2005. The

comparable statewide MIR for *Oc. triseriatus* sampled from New York and Ohio was 0.15 and 0.92 respectively,[14,28] suggesting qualitative differences in transmission intensity. Nonetheless, entomological estimates of transmission risk must also consider other parameters including the relative abundance of the vector. While our study establishes the presence of LACV in New England, additional sampling of *Oc. triseriatus* and other vector species is required to accurately determine the local transmission risk of this virus.

Our infrequent detection of LACV in CT may also stem from under-sampling of *Oc. triseriatus* by our trapping methods. Mosquitoes were collected using CO_2-baited CDC light traps and gravid traps, which are not efficient for luring and trapping *Oc. triseritatus*.[29] Collection of *Oc. triseriatus* by means of human bait or vacuuming the vegetation may be more effective,[30,31] but these methods are relatively time consuming and impractical for operational surveillance at a large number of trapping locations. The problems associated with conventional trapping methods has led to the adoption of ovitraps as the principle method for monitoring *Oc. triseriatus* populations.[32] Ovitraps were used to estimate the prevalence of LACV infection in mosquitoes sampled from emerging foci in North Carolina,[33,34] Tennessee,[33] West Virginia,[35] and Virginia[36] and these traps were shown to be highly effective and selective for *Oc. triseriatus* sampled in CT.[37] This approach, however, detects LACV infections acquired solely by TOT and may be less effective for surveillance in New England when considering vector competence studies. Geographic strains of *Oc. triseriatus* from CT and Massachusetts were shown to be less permissive to TOT than those from midwestern United States,[38] perhaps resulting in lower infection rates among field-collected mosquito eggs. These findings must be extended using the CT isolate of LACV to evaluate whether TOT is more efficient in sympatric viral-vector pairs.

Our isolation of LACV from New England mosquitoes has important public health implications. The isolate was derived from a densely populated region of CT and it is possible that this virus is more broadly distributed throughout this region. To date, only 2 cases of California group encephalitis have been documented among residents of CT. One case was attributed to Jamestown Canyon virus infection[39] and the other was probably acquired outside of the state (CT Department of Health, personal communication). Despite the paucity of human cases in this region, health care providers should consider LAC encephalitis as a possibility among residents of New England when arboviral etiology is suspected.

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