Am. J. Trop. Med. Hyg., 93(1), 2015, pp. 11–17
doi:10.4269/ajtmh.15-0132

Emergence of a New Lineage of Cache Valley Virus (*Bunyaviridae: Orthobunyavirus*) in the Northeastern United States

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Abstract. Cache Valley virus (CVV; Family *Bunyavidae*, Genus *Orthobunyavirus*) is a mosquito-borne zoonosis that frequently infects humans and livestock in North and Central America. In the northeastern United States, CVV transmission is unpredictable from year-to-year and may derive from the periodic extinction and reintroduction of new virus strains into this region. To evaluate this possibility, we sequenced and analyzed numerous CVV isolates sampled in Connecticut during an 18-year period to determine how the virus population may change over time. Phylogenetic analyses showed the establishment of a new viral lineage during 2010 that became dominant by 2014 and appears to have originated from southern Mexico. CVV strains from Connecticut also grouped into numerous sub-clades within each lineage that included viruses from other U.S. states and Canada. We did not observe the development and stable persistence of local viral clades in Connecticut, which may reflect the episodic pattern of CVV transmission. Together, our data support the emergence of a new lineage of CVV in the northeastern United States and suggest extensive dispersal of viral strains in North America.

INTRODUCTION

Cache Valley virus (CVV; family *Bunyaviridae*, genus *Orthobunyavirus*) occurs throughout much of North and Central America where the virus circulates in an enzootic cycle involving mammal-biting mosquitoes and deer hosts.[1–4] Humans are frequently exposed to the virus in endemic regions with antibody prevalence ranging from 3% to 19%.[2,5–7] The development of severe disease occurs rarely in humans but is potentially life-threatening with symptoms ranging from fever to encephalitis.[8–10] In addition, CVV is responsible for epizootics in sheep, causing fetal death and congenital defects in these animals.[11–13]

CVV includes a number of regional subtypes and varieties based on antigenic and genetic relationships. The virus is closely related to but distinct from a number of viruses circulating in South and Central America and Mexico including Maguari virus, Xingu virus, Cholul virus, and Fort Sherman virus.[14–17] CVV may be further delineated into regional varieties that include Tlacotalpan virus from Veracruz, Mexico that is genetically distinct from strains circulating in the United States and Canada.[18] Phylogenetic comparisons indicate that CVV strains from United States and Canada segregate into a single genetically conserved lineage that has persisted over a 60-year period.[18,19] Viruses grouped into sub-clades within this lineage but without clear evidence of spatial or temporal genetic structure. Nevertheless, firm conclusions could not be drawn about the phylogeography because prior analyses were based on a limited number of viral sequences.

In Connecticut, CVV is detected in mosquitoes during most years of arbovirus surveillance but virus activity is highly variable and not directly associated with mosquito abundance.[20] The reasons for these oscillations remain unclear but may derive from the periodic extinction and re-introduction of new virus strains into this region. Accordingly, we sequenced and analyzed numerous virus isolates sampled over an 18-year period in Connecticut to determine how the virus population may change over time. Specifically, portions of the small (S), medium (M), and large (L) genomic segments of CVV were sequenced and phylogenetic relationships were evaluated to differentiate strains, monitor their regional persistence, and compare with viruses from other geographic regions.

MATERIALS AND METHODS

Virus strains. The majority of CVV isolates analyzed in this study ($N = 70$) were obtained from mosquitoes collected during the statewide surveillance program in Connecticut. Mosquitoes were trapped at 91 locations statewide from June to October of 1997–2014 and processed for virus isolation in Vero, clone E6 cell cultures as previously described.[20] CVV was isolated during 11 of the 18 years of mosquito testing and representative viruses were selected from each of these years in 50 locations throughout Connecticut (Figure 1). The number of viral isolates sequenced from each year is as follows: 1998 ($N = 9$), 2000 ($N = 1$), 2001 ($N = 2$), 2003 ($N = 18$), 2005 ($N = 2$), 2006 ($N = 2$), 2008 ($N = 5$), 2009 ($N = 1$), 2010 ($N = 5$), 2011 ($N = 13$), and 2014 ($N = 12$), which reflects fluctuating levels of virus activity among years.[20] We also sequenced six CVV strains obtained from North Dakota during 2005,[21] an earlier CVV isolate from Connecticut in 1979,[22] and the prototype strain of Playas virus from Ecuador in 1975.[23] Portions of the S and L segments were sequenced from viral strains from Manitoba, Canada 1981 ($N = 1$), New Jersey 1982 ($N = 1$), and Oregon 1969 ($N = 1$). Additional sequences were obtained from GenBank and included CVV strains from other locations in United States, Canada, Mexico, and Panama.

Reverse transcription-polymerase chain reaction and nucleotide sequencing. Viral RNA was isolated from the primary virus cultures using the QIamp viral RNA Kit (Qiagen, Valencia, CA). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the Titan One-Tube RT-PCR System (Roche Diagnostics, Indianapolis, IN) and using PCR primers targeting the three genomic segments of CVV (Table 1). Primers BUNS+ new and BUNS– new were used to amplify the S segment under the following thermal cycling conditions: 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

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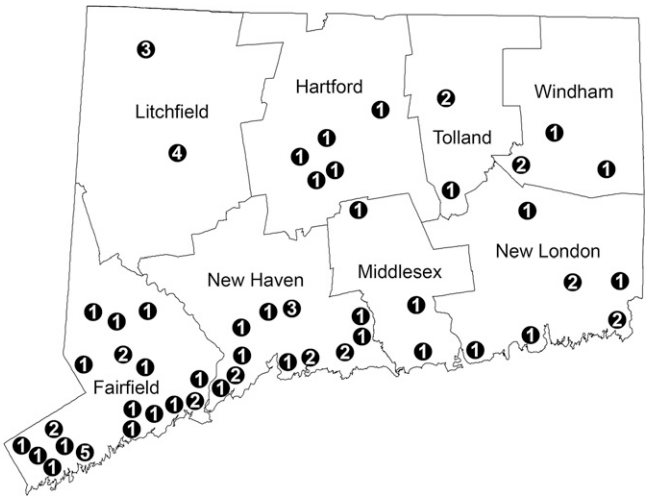

FIGURE 1. County map of Connecticut showing the geographic location and number of Cache Valley virus isolates analyzed in this study.

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. The entire M segment was amplified using primers M14C and M4510R 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. Primers targeting a portion of the M segment (M14C and CVM759r) and L segment (BUNL15C and CVLrev) were used under the same thermal cycling conditions described for the entire M segment except the 68°C extension step was set at 1 minute instead of 4 minutes. Amplification products of the appropriate size were purified using the PCR purification kit (Qiagen, Valencia, CA) and commercially sequenced (Keck Center, New Haven, CT) using primers listed in Table 1.

Genetic analyses. Overlapping sequence chromatograms were edited using ChromasPro (Technelysium Ltd, Tewantin, Australia) and edited nucleotide sequences were deposited in GenBank (accession numbers KP835801–KP835938). Multiple sequence alignments were generated by the ClustalW algorithm. Nucleotide alignments were then manually inspected and coding sequences were translated into protein to check the position of the open reading frame in the alignment. Four data sets were generated for phylogenetic analysis: 1) M segment—1,803 nucleotide sites encoding the Gc protein, 2) M segment—525 nucleotides of the Gn protein gene, 3) S segment—757 nucleotides of the nucleocapsid gene and 3′ untranslated region, and 4) L segment—528 nucleotides of the 5′ untranslated region and RNA polymerase gene. Phylogenetic relationships were evaluated by maximum-likelihood analysis in Mega 6.0.[24] The optimal nucleotide substitution model for each data set was selected by performing ML fits of 24 different models in Mega. Support for individual nodes was evaluated by performing 1,000 bootstrap replicates.

RESULTS

To evaluate phylogenetic relationships of CVV strains, we analyzed a 1,803 nucleotide region of M segment encoding the Gc protein. The final alignment included 71 viral sequences from Connecticut, 20 from other U.S. states and Canada, 1 from Mexico, and 3 from Central and South America. CVV sequences segregated into two distinct and well-supported groups, each with 100% bootstrap support, by maximum-likelihood analysis (Figure 2). Lineage 1 comprised CVV sampled from United States and Canada during 1952–2011, a human isolate from Panama, and Playas virus from Equator. More recent CVV strains from Connecticut, New Jersey, and New York 2010–2014 clustered together and grouped with Tlacotalpan virus from Mexico to form lineage 2. Connecticut viruses also grouped into numerous sub-clades within each lineage. These clades were not defined by in-state location, year of isolation, or mosquito species and sometimes included viruses from other U.S. states and Canada. Our data support the emergence of a new genotype of CVV in the northeastern United States and suggest extensive dispersal of viral strains in North America.

We then analyzed another region of the M segment encoding the Gn protein to facilitate the inclusion of more CVV sequences from Mexico. Figure 3 shows the phylogenetic

TABLE 1
Primers used for RT-PCR and sequencing reactions

Segment	Primer name	Sequence (5′–3′)*	Genomic position	Used for	PCR amplicon size (nt)
S	BUNS+ new	*TGACC* AGTAGTGTACTCCAC	1–15	PCR	960
	BUNS– new	*CAAGC* AGTAGTGTGCTCCAC	935–950	PCR	–
	BUNS+	AGTAGTGTACTCCAC	1–15	Sequencing	–
	BUNS–	AGTAGTGTGCTCCAC	935–950	Sequencing	–
	BUNSPA1	CTGTCCCCTACCACCCACCCA	863–883	Sequencing	–
M	M14C	*CGGAATTC* AGTAGTGTACTACC	1–14	PCR, sequencing	767
	CVM759r	CGGCATGAAAATTGGCATCA	740–759	PCR, sequencing	–
	M4510R	*ATCGCGT* AGTAGTGTGCTACC	4450–4463	PCR	4478
	CVMfwdC	ACAAAAGAATGCCATAATGC	2269–2288	Sequencing	–
	CVMfwdD	CTTTGGTGAGTATTGTATATCTC	2679–2701	Sequencing	–
	CVMrev-1	AATTCCACCTAATGCAGGGAT	2980–3000	Sequencing	–
	CVMfwdE	CACACAACATGATGAGCACTG	3129–3149	Sequencing	–
	CVMfwdF	CTCAATCCTAGGCATGGG	3561–3578	Sequencing	–
	CVMrevB	CTGGTCCCCTGTGTTCACTTC	4081–4101	Sequencing	–
	CVMrevA	CCCCTCATCTCTAACCCTGCA	4147–4167	Sequencing	–
L	BUNL15C	*CGGCC* AGTAGTGTACTCCTA	1–15	PCR, sequencing	643
	CVLrev	TCATCCATACACCATGGTGCTGT	616–638	PCR, sequencing	–

L = large; M = medium; S = small; RT-PCR = reverse transcription-polymerase chain reaction.
*Nonviral sequences are indicated in italics.

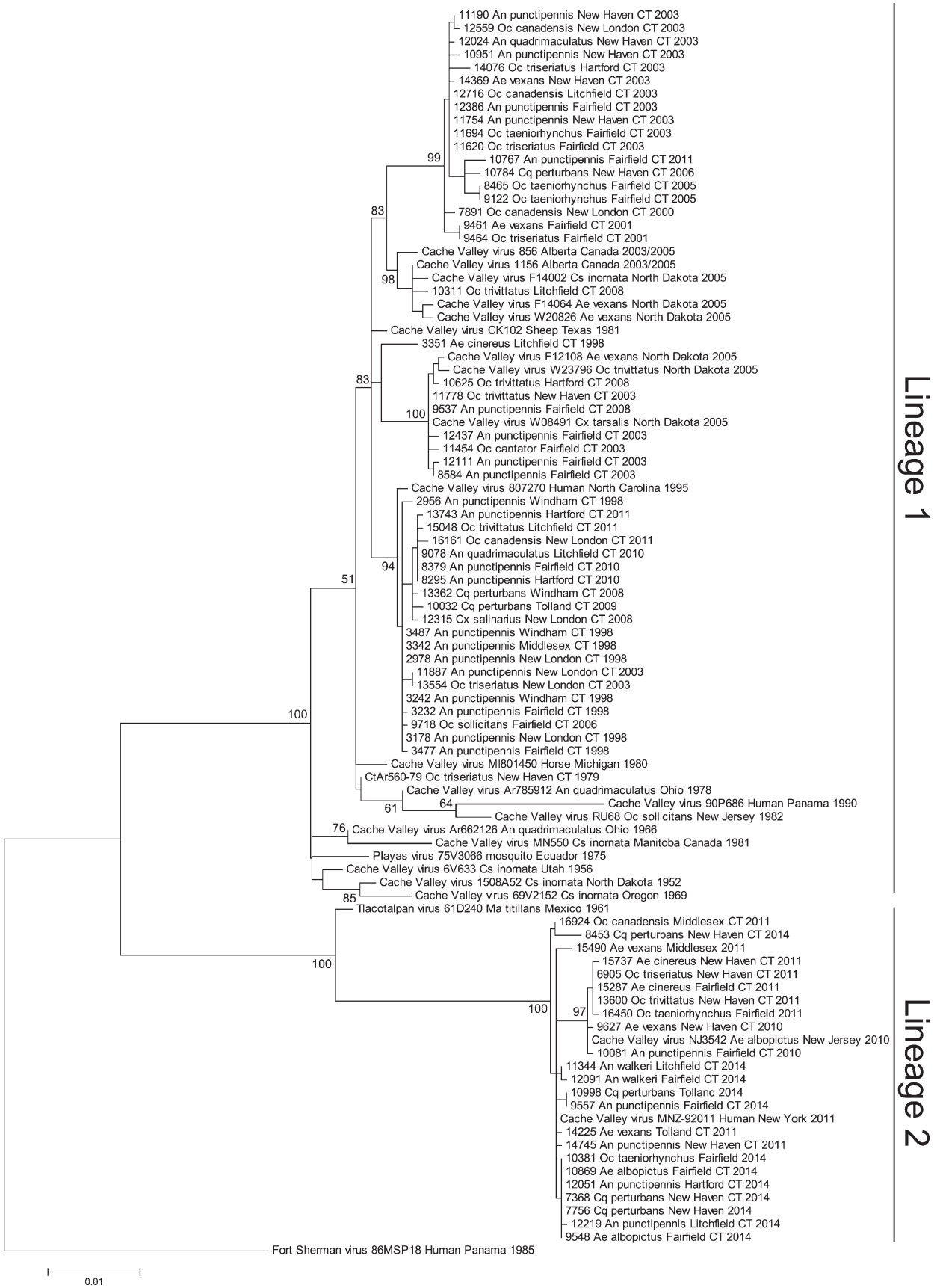

FIGURE 2. Maximum-likelihood tree of M segment sequences encoding the Gc protein. Taxon names for Connecticut strains specify the strain number, mosquito species, county where they were collected, and year of isolation. Number at nodes indicate bootstrap support for values > 50%. Branch lengths are proportional to the number of nucleotide substitutions.

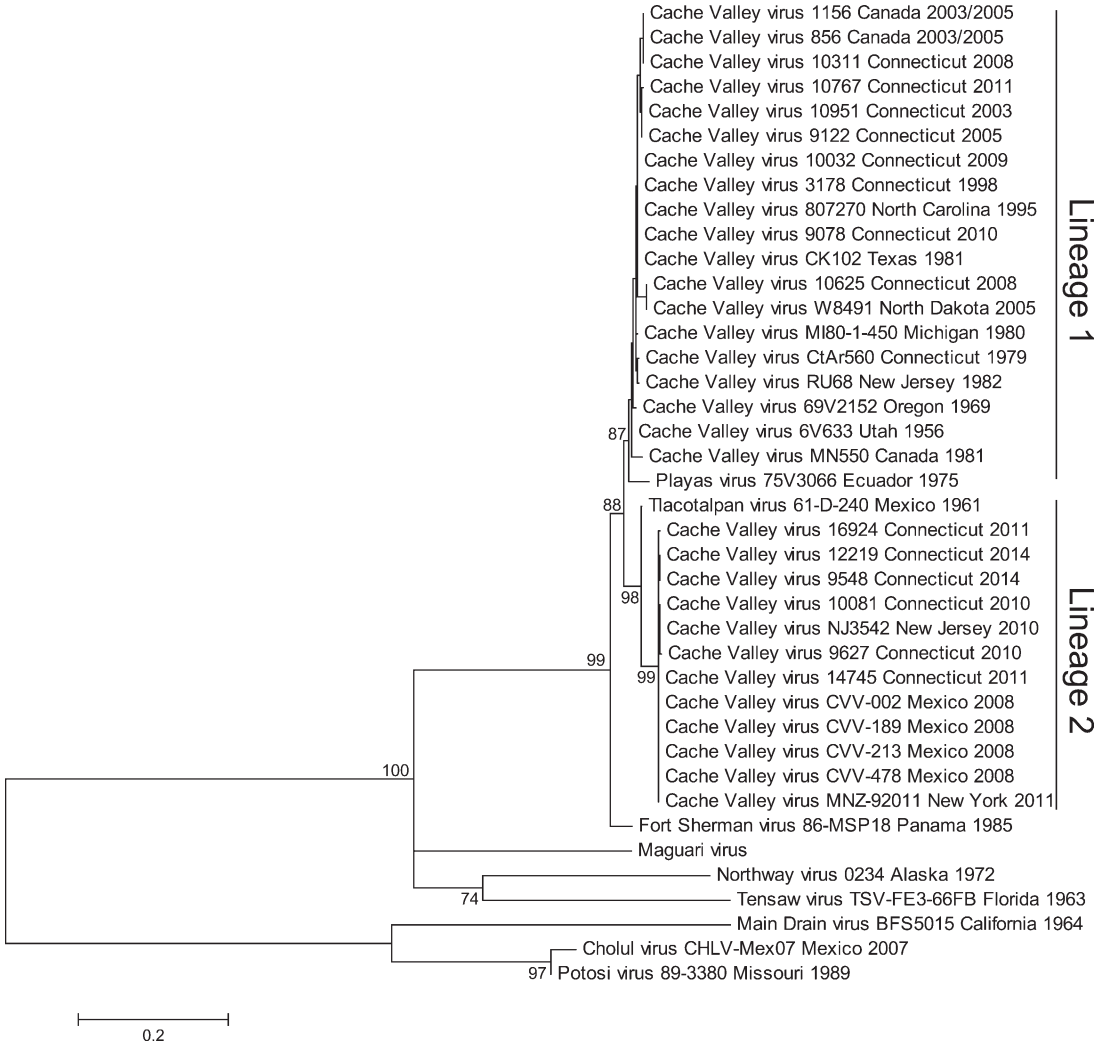

FIGURE 3. Maximum-likelihood tree of medium (M) segment sequences encoding the Gn protein. Number at nodes indicate bootstrap support for values > 50%. Branch lengths are proportional to the number of nucleotide substitutions.

relationships of 40 viral sequences analyzed by maximum-likelihood analysis. We included more distant outgroup taxa in this analysis, which obscured fine resolution of CVV relationships; nevertheless, lineages 1 and 2 could be distinguished in the analysis with 87% and 98% booststrap support, respectively. Lineage 2 comprised the same strains from Connecticut, New Jersey, and New York in the earlier analysis and these strains were most similar to viruses isolated in Yucatan, Mexico during 2008.

CVV has the capacity to undergo reassortment by exchanging whole genomic segments with heterologous viruses during mixed infections. To determine whether lineage 2 viruses emerged due to genetic reassortment with another virus, we sequenced and analyzed portions of the S and L segments by maximum-likelihood methods. The topologies of S- and L-segment trees (Figures 4 and 5) differed somewhat from each other and the M segment tree (Figure 3). The position of Cholul virus, Ft. Sherman virus, and Potosi were inconsistent among trees, which had been previously reported as evidence for RNA segment reassortment.[17,25] Phylogenetic relationships among CVV strains, however, were consistent in all three trees generated from the S, M, and L segments. The S segment tree shows two distinct groups of CVV that correspond to lineage 1 with 96% bootstrap support and lineage 2 from previous analyses (Figure 4). Phylogenetic reconstruction of the L segment revealed strong bootstrap support (100%) for lineage 2 but not lineage 1 (Figure 5). We conclude that lineage 2 viruses form a monophyletic group and are not reassortant viruses.

Our analyses indicated that all of the viral sequences from Connecticut 2014 belonged exclusively to lineage 2 as shown in Figure 2. To determine whether lineage 2 may have supplanted lineage 1, we genotyped the remaining strains (N = 13) that were collected during statewide surveillance during 2014 by sequencing the Gn gene. These viruses were identical or most similar to lineage 2 viruses from northeastern United States and Yucatan, Mexico (99.4–100% sequence identity).

DISCUSSION

Our study documents the emergence of a new lineage of CVV in the northeastern United States that shares recent common ancestry with viral strains from southern Mexico. Lineage 2 viruses first appeared in Connecticut and New Jersey in 2010 and were most similar to viruses isolated 2 years earlier in Yucatan, Mexico. This suggests long-distance

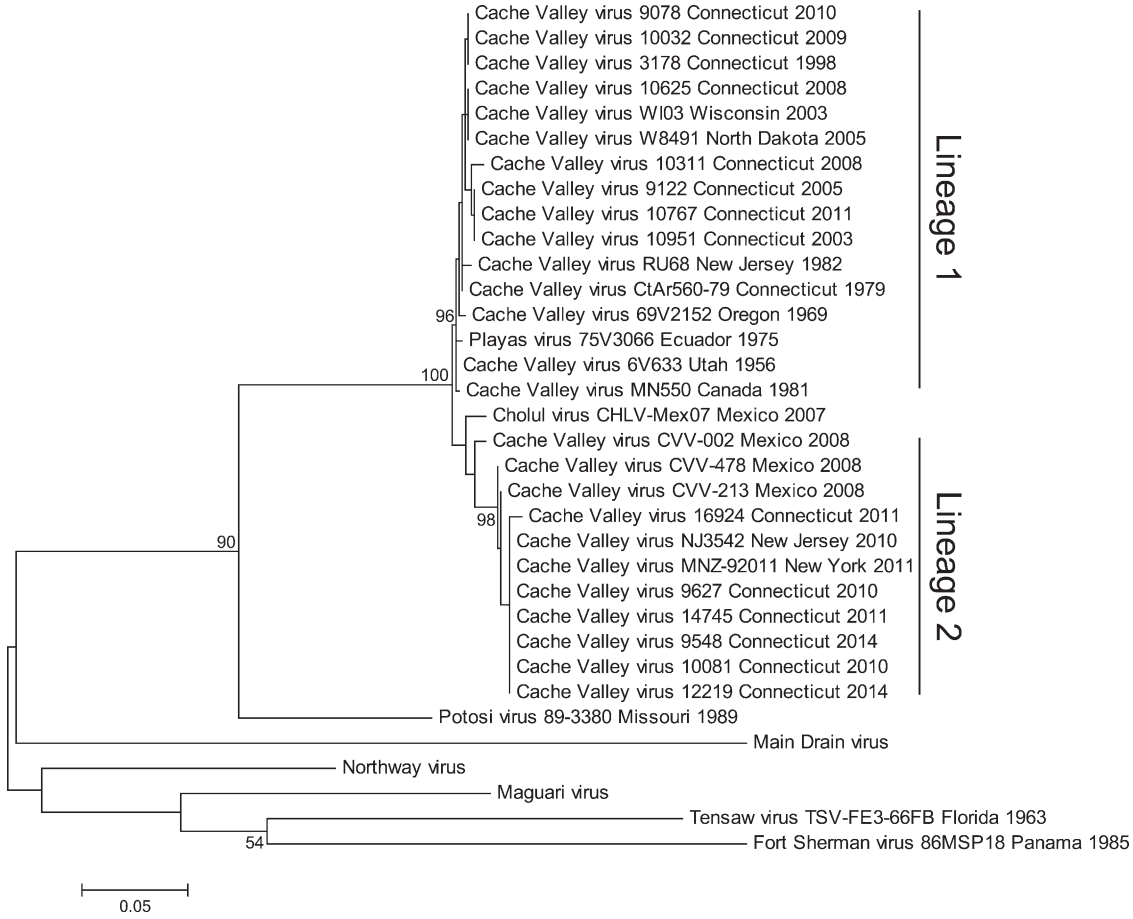

FIGURE 4. Maximum-likelihood tree of small (S) segment sequences. Number at nodes indicate bootstrap support for values > 50%. Branch lengths are proportional to the number of nucleotide substitutions.

dispersal of viral strains from Mexico into northeastern United States; however, the pathway for migration is unclear based on our current sample of viruses. It is also possible that lineage 2 viruses originated from another region not represented in this analysis. CVV is known to occur in southern Canada, Mexico, Panama, and throughout the continental United States except in southeastern states where related Tensaw virus is prevalent.[1] Our sample includes CVV strains from across much of its geographic range, extending from Oregon to Connecticut and from Canada to Panama, but sampling is very limited in most geographic regions. Rather, this study focuses primarily on viral population change at the statewide level over many consecutive years and clearly shows the recent introduction and establishment of a new lineage in the northeastern United States.

The phenomenon of lineage replacement has been well documented for a number of different arboviruses including dengue virus and West Nile virus.[26–30] This occurs when a new viral lineage or genotype is introduced into a region and rapidly displaces its native counterpart. The mechanistic basis of lineage replacement is not always clear but could stem from differences in viral fitness or may reflect stochastic processes such as random genetic drift.[29,31–34] In this study, we found that all of the CVV isolates collected during 2014 belonged exclusively to lineage 2 indicating a possible lineage replacement event; however, future monitoring is required to determine whether lineage 2 becomes permanently fixed. Moreover, the impact of lineage replacement is not clear as viruses from both lineages have the capacity to cause human disease and infect a wide diversity of mosquito species.

Our sequence analysis included 70 CVV strains collected during 18 years of continuous statewide arbovirus surveillance in Connecticut. These viruses grouped into a number of well-supported sub-clades within each major lineage but there was no clear segregation of viruses by geographic location, year of isolation, or mosquito species. Many of these sub-clades contained viruses from other U.S. states and Canada indicating widespread dispersal of viral strains in North America. These findings differ from that of another Bunyavirus—Jamestown Canyon virus that also perpetuates in a deer-mosquito cycle in this region. Our prior analyses found that Jamestown Canyon virus could be distinguished into two major lineages that persisted in different regions in Connecticut, suggesting geographic isolation of this virus.[35] Unlike CVV, Jamestown Canyon virus is consistently detected every year during statewide surveillance and the virus may overwinter locally in mosquito eggs infected by vertical transmission.[36–38] These factors might contribute to the stability of Jamestown Canyon virus populations in different regions of the state as compared with CVV that occurs more sporadically.

CVV proved to be genetically conserved throughout its geographic range over a 60-year sampling period. Mean nucleotide distances between lineages 1 and 2 ranged from 3.1% for the S segment sequences to 7.0% for M segment

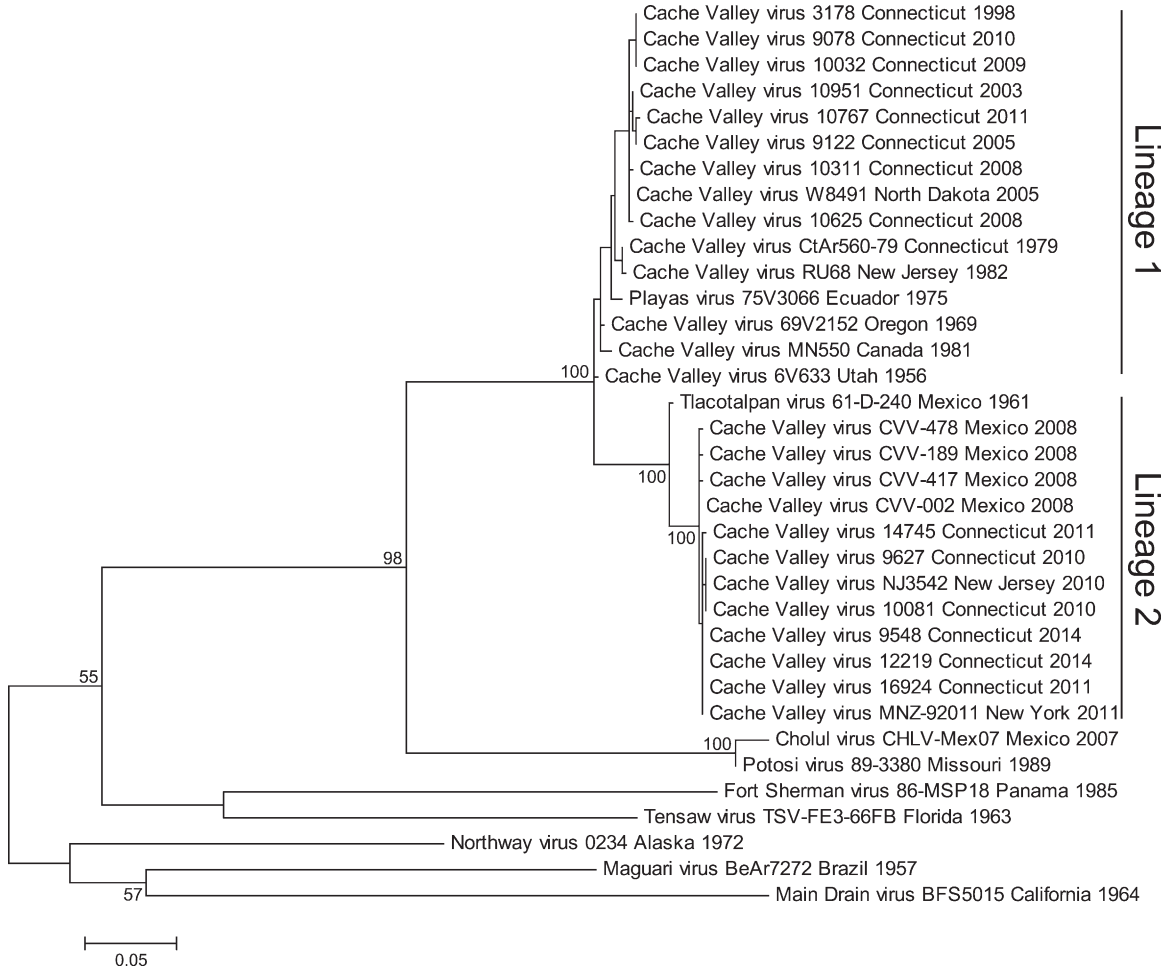

FIGURE 5. Maximum-likelihood tree of large (L) segment sequences. Number at nodes indicate bootstrap support for values > 50%. Branch lengths are proportional to the number of nucleotide substitutions.

sequences encoding the Gc protein. For comparison, nucleotide distances were from 5.7% to 12.4% between Jamestown Canyon virus lineages and were up to 14.6% for La Crosse virus lineages circulating in the United States.[35,39] This suggest that CVV lineages diverged relatively recently from a common ancestor; however, it is also possible that nucleotide substitution rates were lower for CVV than for Jamestown Canyon virus and La Crosse virus. We cannot differentiate between these possibilities based on this analysis.

Our sequence data suggest that CVV and Playas virus are not distinct viruses as previously proposed.[23] Playas virus from Ecuador was indistinguishable from CVV and grouped within other North American strains to form lineage 1 based on phylogenetic analysis of S, M, and L sequences. These results conflict with serological classification by neutralization tests that targets glycoproteins encoded on the M segment.[23] We analyzed portions of the Gc (Figure 2) and Gn proteins (Figure 3) but there were no obvious differences in encoded proteins to explain antigenic differences among viruses. Full genome sequencing might help resolve this discrepancy; nevertheless, our initial analyses of S, M, and L sequences clearly show that CVV and Playas virus are closely related to each other.

In conclusion, we tracked the geographic distribution and persistence of CVV variants in Connecticut over an 18-year sampling period. We show the emergence of a new viral lineage that included strains isolated from southern Mexico. Phylogenetic patterns show wide circulation of CVV strains across a broad geographic region. We did not observe the stable persistence of local variants of CVV in Connecticut, which may reflect the episodic patterns of CVV transmission. Multiple clades often appeared in a specific year indicating that a number of virus variants may emerge and circulate simultaneously when conditions support CVV amplification.

Received February 13, 2015. Accepted for publication March 28, 2015.

Published online May 11, 2015.

Acknowledgments: We thank Angela Bransfield, Shannon Finan, and Michael Misencik for their technical assistance in the isolation and sequencing of viruses from field-collected mosquitoes. We also thank Terrill Goodman, Michael Misencik, John Shepard, Michael Thomas, and Michael Vasil for the collection and identification of mosquitoes for virus testing.

Financial support: This work was supported in part by grants from the Centers for Disease Control and Prevention (U50/CCU116806-01-1), the U.S. Department of Agriculture (58-6615-1-218, CONH00768 and CONH00773).

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