Insect-Specific Flaviviruses from *Culex* Mosquitoes in Colorado, with Evidence of Vertical Transmission

Bethany G. Bolling,* Lars Eisen, Chester G. Moore, and Carol D. Blair

Arthropod-Borne and Infectious Diseases Laboratory, Department of Microbiology, Immunology, and Pathology,
Colorado State University, Fort Collins, Colorado

Abstract. Mosquitoes were collected in Colorado during 2006 and 2007 to examine spatial and seasonal patterns of risk for exposure to *Culex* vectors and West Nile virus. We used universal flavivirus primers to test pools of *Culex* mosquitoes for viral RNA. This led to the detection and subsequent isolation of two insect-specific flaviviruses: *Culex* flavivirus (CxFV), which was first described from Japan, and a novel insect flavivirus, designated Calbertado virus (CLBOV), which has also been detected in California and Canada. We recorded both viruses in *Cx. tarsalis* and *Cx. pipiens* from Colorado. Furthermore, quantitative reverse transcription polymerase chain reaction (RT-PCR) revealed the presence of CxFV RNA in *Cx. pipiens* eggs and larvae from a laboratory colony established in 2005 and naturally infected with CxFV, suggesting vertical transmission as a means of viral maintenance in natural *Culex* populations. Finally, we present phylogenetic analyses of the relationships between insect-specific flaviviruses and other selected flaviviruses.

INTRODUCTION

The genus *Flavivirus* contains numerous arthropod-borne viruses (arboviruses) that are associated with disease in vertebrates. These arboviruses are capable of replicating in both vertebrate and invertebrate cells.¹ There is, however, a group of viruses within the genus *Flavivirus* that seems to replicate only in invertebrate cells. These are considered insect-specific flaviviruses and include cell-fusing agent virus (CFAV),² Kamiti River virus (KRV),^{3,4} *Culex* flavivirus (CxFV),⁵ Quang Binh virus,⁶ *Aedes* flavivirus (AeFV),⁷ Nounané virus (NOUV),⁸ Lammi virus (LAMV),⁹ and Nakiwogo virus.¹⁰ Phylogenetic analyses suggest that insect-specific viruses are the most divergent group within the genus *Flavivirus* and may represent the earliest forms of flaviviruses.^{5,11}

CFAV, the first insect-specific flavivirus described, was isolated from a cultured line of *Ae. aegypti* Linnaeus mosquito cells.² It has recently been isolated from field-collected mosquitoes in Puerto Rico.¹¹ KRV was isolated in 2003 from *Ae. macintoshi* Marks larvae and pupae from Kenya and described as a CFAV-related flavivirus.^{3,4} *Culex* flavivirus was first isolated in Japan from *Cx. pipiens* Linnaeus and other *Culex* spp. mosquitoes⁵ and has since been described in *Culex* spp. mosquitoes from Guatemala,¹² Mexico,¹³ Trinidad,¹⁴ Texas,¹⁴ Iowa,¹⁵ and Uganda.¹⁰ Quang Binh virus was isolated from *Cx. tritaeniorhynchus* Giles collected in Vietnam in 2002.⁶

Aedes flavivirus, isolated from *Aedes* spp. mosquitoes in Japan, groups with other insect-specific flaviviruses in phylogenetic analyses but interestingly, has a high degree of similarity to cell silent agent (CSA), which is a flavivirus-related nucleotide sequence found integrated into the genome of *Ae. albopictus* Skuse.^{7,16} Two other recently described flaviviruses, NOUV⁸ and LAMV,⁹ seem to represent a distinct group of insect-specific flaviviruses. Phylogenetic analyses group them with mosquito-borne flaviviruses that cause disease in vertebrates, such as West Nile and Japanese encephalitis viruses, but they do not seem to grow in vertebrate cells.^{8,9} Research on insect-specific flaviviruses is rapidly expanding, as new viruses are being isolated and characterized.

The findings described here originated from adult mosquito collections conducted along riparian corridors in northeastern Colorado during 2006 and 2007 to investigate spatial and temporal risk patterns for exposure to West Nile virus (WNV)-infected *Culex* spp. mosquitoes. During the course of this study, we tested mosquito pools by reverse transcription polymerase chain reaction (RT-PCR) using universal flavivirus primers and detected non-WNV RNA sequences, which prompted this research to determine the identity of these RNA sequences and describe their seasonal dynamics.

MATERIALS AND METHODS

Study area. The five-county study area (Larimer, Weld, Morgan, Logan, and Washington counties) is located in northeastern Colorado and has been previously described.¹⁷ Briefly, mosquito sampling was conducted along three rivers: the Cache la Poudre River and the Big Thompson River, which both emerge from the Rocky Mountains in western Larimer County, and the South Platte River, into which the other two rivers merge in the eastern Colorado plains (Figure 1). Mosquito collections were conducted in plains, foothills, and montane areas along these riparian corridors in sampling sites that could be accessed by automobile. Site locations were mapped with a global positioning system (GPS) receiver (Trimble Geo XT; Trimble Corp., Sunnyvale, CA) and visualized using ArcGIS 9.3 (ESRI, Redlands, CA).

Mosquito collection and identification. Mosquitoes were collected using CO₂-baited Centers for Disease Control and Prevention (CDC) miniature light traps (John W. Hock Company, Gainesville, FL) that were suspended ~1.5 m above the ground and operated from afternoon (1500–1700 hours) until morning (0800–1000 hours). Sampling sites contained two traps baited with ~1 kg dry ice, and they were located directly along the aforementioned rivers. Sampling during the summer of 2006 included 10 sites located along the Cache la Poudre River and 1 site by the Dixon Reservoir in Fort Collins (Figure 1). This spanned an elevation gradient from below 1,600 m in Fort Collins up to 2,360 m in the Poudre Canyon. The sites were sampled every 2 weeks from April 10 to October 27, 2006.

Sampling in 2007 included 20 sites along the Big Thompson and South Platte rivers and two additional sites located south

* Address correspondence to Bethany G. Bolling, Division of Vector-Borne Diseases, Centers for Disease Control and Prevention, 3150 Rampart Rd., Fort Collins, CO 80521. E-mail: bbolling@cdc.gov

FIGURE 1. Location of mosquito sampling sites for 2006 (along the Cache la Poudre River) and 2007 (along the Big Thompson River and South Platte River). The location of the targeted five-county area in Colorado is shown in the inset map.

of the Big Thompson River in the Loveland area (Figure 1). This included an elevation gradient ranging from 1,215 m in the prairie landscape of eastern Colorado to 1,840 m in the montane habitat of the Big Thompson Canyon. The sites were sampled every 2 weeks beginning on June 20 and continuing through September 14, 2007. Collected mosquitoes were examined with a dissecting microscope and identified to species using morphological characters.^{18,19}

Detection of flavivirus RNA and nucleotide sequencing.

Culex mosquitoes were examined for presence of viral RNA by RT-PCR following the method of Bolling and others,²⁰ with the modifications outlined below. Mosquitoes were identified on a chill table and placed in pools of 1–50 by species, sex, site, trap location, and date. Mosquito pools were then stored at –70°C until processed for viral RNA detection.

Each pool was triturated for 45 seconds with a vortex mixer in a 5-mL round-bottom polypropylene tube (Becton Dickinson, Franklin Lakes, NJ) using 1.5 mL diluent (minimum essential medium [MEM] containing 2% fetal bovine serum [FBS] and 100 µg/mL penicillin/streptomycin supplemented with L-glutamine and non-essential amino acids) and four copper-coated steel shot (4.5-mm diameter and 0.177-in caliber). Suspensions were centrifuged at 3,000 rpm for 10 min at 4°C. Total RNA was extracted from 140 µL supernatant using the QIAamp viral RNA Mini kit (Qiagen Inc., Valencia, CA). RNA was eluted in 60 µL nuclease-free water (Ambion Inc., Austin, TX).

RT-PCR was carried out using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and GoTaq DNA polymerase (Promega, Madison, WI). Mosquito pools were first tested using universal flavivirus primers (cFD2 and MAMD) targeting a portion of the NS5 gene.²¹ Pools testing positive for flavivirus RNA were tested with virus-specific primers (Calbertado virus [CLBOV] primer sequences available on request).^{5,22,23} PCR products were visualized after electrophoresis on a 1% agarose gel stained with ethidium bromide. Negative (no template) and positive controls were included in each RT-PCR.

Infection rates per 1,000 individuals were calculated as bias-corrected maximum likelihood estimates using the Excel Add-In PooledInfRate, version 3.0.²⁴ Presented infection rates are based on males and females combined. The most common

Culex spp. found in our light-trap collections were *Cx. tarsalis* Coquillett and *Cx. pipiens*. Because *Cx. tarsalis* are easily identifiable, even when badly damaged, mosquitoes identified only as *Culex* spp. were grouped with *Cx. pipiens* for analyses.

For nucleotide sequencing, RT-PCR was carried out as described above using primers FU1 or FU2 and cFD3.²⁵ The PCR products were purified using a QIAquick Gel Extraction kit (Qiagen Inc., Valencia, CA) and sequenced on an ABI 3130 Genetic Analyzer. Sequences obtained for the NS5 genome region were compared with other flavivirus sequences using the BLASTn program (blast.ncbi.nlm.gov/Blast.cgi).²⁶

Phylogenetic analysis. Nucleotide sequences of insect-specific flaviviruses detected from *Culex* spp. in Colorado were compared with homologous flavivirus sequences as follows. The sequences were aligned using ClustalX 1.81.²⁷ Phylogenetic trees were constructed using neighbor-joining, maximum parsimony, and maximum likelihood analyses in PAUP 4.0²⁸ as well as Bayesian analysis using MrBayes 3.1.²⁹ Resulting trees were midpoint-rooted using RETREE from the Phylip package³⁰ and were displayed using Treeview 1.6.6.³¹

Virus isolation. To isolate viruses, 100 µL homogenate supernatants from infected pools were inoculated onto Vero cells (African green monkey kidney), DF-1 cells (chicken embryo fibroblast), and C6/36 cells (*Ae. albopictus*) in 25-cm² flasks. After addition of 1 mL medium, the flasks were rocked for 1 hour at room temperature; 4 mL medium were then added, and cells were monitored daily for cytopathic effects (CPE). The Vero and DF-1 cells were maintained at 37°C and 7% CO₂ with MEM supplemented with 10% FBS, penicillin/streptomycin, L-glutamine, and non-essential amino acids. The C6/36 cells were maintained at 28°C with L-15 medium supplemented with 7% FBS, penicillin/streptomycin, L-glutamine, and non-essential amino acids. Cell culture medium was harvested after each passage for four passages and tested by RT-PCR to assess virus propagation.

Immunofluorescence assay. Indirect fluorescent antibody assays (IFA) were conducted on spot slides of C6/36 cells infected with either CxFV or CLBOV using polyclonal antibodies against Japanese encephalitis virus (JEV), St. Louis encephalitis virus (SLEV), WNV, CxFV, or CLBOV. Murine hyperimmune ascitic fluids for JEV (M30178ABY), SLEV (VS0102), and WNV (M30200ABY) were obtained from the

Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO. Virus-specific antisera for the Colorado strains of CxFV and CLBOV were produced by subcutaneous immunization of imprinting control region (ICR) mice with infected C6/36 cell culture medium clarified by centrifugation. Mice were immunized three times at 2-week intervals with complete Freund's adjuvant for the first immunization and incomplete Freund's adjuvant for the remaining immunizations. The use of all animals was reviewed and approved by the Animal Care and Use Committee at Colorado State University (Animal Protocol 08-067A-03). An indirect enzyme-linked immunosorbent assay (ELISA) of mouse sera 2 week after the final immunization confirmed antibody production. Serum was cross-absorbed two times to sonicated, uninfected C6/36 cells to reduce non-specific binding. Secondary antibody for IFA was biotinylated sheep anti-mouse immunoglobulin G (IgG) followed by streptavidin-fluorescein (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

Persistently CxFV-infected mosquito colony. A *Cx. pipiens* colony was established from egg rafts collected in Fort Collins during the summer of 2005. After the discovery of CxFV and CLBOV in local field populations, the colony was determined to be infected with CxFV by RT-PCR as described above and virus isolation in C6/36 cells. Mosquito homogenates taken from the colony in 2005 and stored at -80°C , tested positive for CxFV, indicating that the mosquitoes were infected at the time that the colony was established. The colony is maintained in a $60 \times 60 \times 60$ -cm cage at 25°C , 75% relative humidity, and a 16 hour to 8 hour, light to dark cycle. To investigate viral maintenance within the colony, RNA extractions were initially performed using a QIAamp viral RNA Mini kit as described above. To increase sensitivity of RNA detection in individual mosquitoes, Trizol (Invitrogen, Carlsbad, CA) was used to extract total RNA from individual egg rafts, individual fourth instar larvae, and individual adult mosquitoes. A quantitative RT-PCR assay was designed using a QuantiTect SYBR Green RT-PCR kit (Qiagen Inc., Valencia, CA) and CxFV-specific primers (available on request).

RESULTS

Discovery of insect-specific flaviviruses in *Culex* spp. In 2006, ~1,300 *Culex* mosquitoes from northeastern Colorado were

tested for viral RNA by RT-PCR using universal flavivirus primers. Unexpectedly, numerous *Culex* pools testing positive for flavivirus RNA were negative for the flaviviruses that most commonly are found in *Culex* in Colorado: WNV and SLEV. To rule out contamination, 80 pools (89 mosquitoes) of *Culiseta* spp. mosquitoes and 64 pools (1,997 mosquitoes) of *Ae. vexans* Meigen were tested by RT-PCR with the universal flavivirus primers. All of these pools tested negative.

PCR products from flavivirus-positive *Culex* pools were then sequenced, revealing similarity in some cases to a previously described insect-specific flavivirus, CxFV. In addition, we discovered sequences from a novel insect-specific flavivirus referred to as Calbertado virus (CLBOV), because this virus has been found in California, Alberta, Canada,^{32,33} and Colorado. Specific primers were designed to detect these insect-specific flaviviruses in mosquito pools.

In 2007, we collected ~43,000 *Culex* spp. mosquitoes. All *Culex* pools were first tested with the universal flavivirus primers. Thereafter, all flavivirus-positive pools were tested with WNV-specific, CxFV-specific, and CLBOV-specific primers.

Trends for insect-specific flaviviruses in *Culex* spp. in time and space. Infection rates for WNV, CxFV, and CLBOV in *Cx. tarsalis* and *Cx. pipiens* were calculated by month (Table 1) and site (Table 2) for both 2006 and 2007. Infection rates include male and female pools of mosquitoes combined, because viral RNA was detected in both. The seasonal patterns of infection rates are shown for 2007 (Figure 2) when *Culex* abundance was higher and virus detection was based on larger numbers of mosquitoes than in 2006.

In the case of the 2006 collections, most CxFV-positive pools (36/37) came from *Cx. pipiens*, with only a single *Cx. tarsalis* pool positive for CxFV. Conversely, all (34) CLBOV-positive pools were from *Cx. tarsalis* (Table 1). This pattern was less clear in 2007, when both CxFV and CLBOV were found in *Cx. tarsalis* as well as *Cx. pipiens*. However, there still was a trend for most CxFV-positive pools being recorded from *Cx. pipiens* (contributing 125 of 178 CxFV-positive pools) and most CLBOV-positive pools coming from *Cx. tarsalis* (contributing 113 of 121 CLBOV-positive pools).

The overall infection rates per 1,000 *Cx. tarsalis* in 2006 were 5.47 for WNV, 0.83 for CxFV, and 40.13 for CLBOV. The corresponding infection rates for *Cx. tarsalis* in 2007 were 2.65 for WNV, 1.34 for CxFV, and 2.95 for CLBOV. Differences

TABLE 1

Monthly infection rates with West Nile virus (WNV), *Culex* flavivirus (CxFV), and Calbertado virus (CLBOV) for *Cx. tarsalis* and *Cx. pipiens* in Colorado from 2006 to 2007

Year	Month	<i>Cx. tarsalis</i>						<i>Cx. pipiens</i>					
		Total mosquitoes	No. of pools	Infection rates (maximum likelihood estimates)				Total mosquitoes	No. of pools	Infection rates (maximum likelihood estimates)			
				WNV*	CxFV	CLBOV				WNV*	CxFV	CLBOV	
2006	April	17	7	0	0	154.98		1	1	0	1,000†	0	
2006	May	4	3	0	0	0		0	0	0	0	0	
2006	June	45	12	0	0	133.48		9	3	0	76.46	0	
2006	July	889	40	7.71	1.12	33.37		56	12	0	247.49	0	
2006	August	189	52	0	0	32.70		43	20	0	638.14	0	
2006	September	77	21	0	0	36.56		18	8	0	777.73	0	
2006	October	0	0	0	0	0		2	1	0	500†	0	
2007	June	7,909	546	0.51	0.25	3.28		110	25	18.94	211.95	17.00	
2007	July	23,420	1,308	1.96	0.86	2.91		568	71	1.72	101.53	3.45	
2007	August	8,662	1,233	6.58	3.31	2.61		1,407	108	1.44	124.35	2.18	
2007	September	444	225	6.80	8.91	2.22		454	38	2.23	67.19	2.15	

Adult mosquitoes were tested in pools of 1–50. Bias-corrected maximum likelihood estimates for infection rates per 1,000 mosquitoes were calculated with Excel Add-In PooledInfRate 3.0.²⁴

*Based in part on data published previously by Bolling and others.¹⁷

†One single pool that was examined tested positive, and therefore, a minimum infection rate was calculated $[(\text{no. of positive pools}/\text{no. of mosquitoes tested}) \times 1,000]$.

TABLE 2

Infection rates by trapping site with West Nile virus (WNV), *Culex flavivirus* (CxFV), and Calbertado virus (CLBOV) for *Cx. tarsalis* and *Cx. pipiens* in Colorado from 2006 to 2007

<i>Cx. tarsalis</i>								<i>Cx. pipiens</i>				
Site	Habitat	Elevation (m)	Total mosquitoes	No. of pools	Infection rates (maximum likelihood estimates)			Total mosquitoes	No. of pools	Infection rates (maximum likelihood estimates)		
					WNV*	CxFV	CLBOV			WNV*	CxFV	CLBOV
Poudre River transect (2006)												
LMP	Plains	1,510	108	18	0	0	45.05	23	11	0	400.08	0
BFH	Plains	1,560	436	37	2.34	2.35	36.95	63	23	0	902.52	0
DIX	Plains	1,585	610	41	9.83	0	37.40	33	7	0	189.75	0
PIR	Foothills	1,610	38	14	0	0	59.40	10	4	0	150.08	0
GAP	Foothills	1,640	10	7	0	0	102.37	0	0	0	0	0
OYG	Montane	1,750	7	7	0	0	0	0	0	0	0	0
STP	Montane	1,860	5	4	0	0	0	0	0	0	0	0
DUG	Montane	2,000	4	4	0	0	0	0	0	0	0	0
EGG	Montane	2,110	1	1	0	0	0	0	0	0	0	0
DAD	Montane	2,130	1	1	0	0	0	0	0	0	0	0
BSW	Montane	2,360	1	1	0	0	0	0	0	0	0	0
Big Thompson River–South Platte River transect (2007)												
OVE	Plains	1,215	1,628	189	8.22	1.25	1.23	60	19	0	33.02	0
ATW	Plains	1,219	2,799	192	2.99	0	0.36	15	9	0	132.11	0
MES	Plains	1,242	3,170	204	1.96	0.32	0.96	24	7	0	0	42.55
COT	Plains	1,272	1,631	178	1.84	1.25	0	115	17	8.32	169.15	0
JEA	Plains	1,286	4,436	242	3.12	0.68	1.39	63	13	17.08	14.92	0
BOY	Plains	1,303	335	124	0	3.04	0	241	14	0	63.56	8.24
WEL	Plains	1,321	4,862	241	2.37	0.21	1.26	40	10	0	27.03	0
SOO	Plains	1,344	3,900	229	3.31	0	1.31	40	10	0	45.88	0
GRE	Plains	1,397	2,035	170	0.98	0.49	3.60	14	8	68.82	235.53	0
MIT	Plains	1,402	4,356	231	1.17	0.93	1.17	137	15	0	173.16	7.06
BRO	Plains	1,418	2,846	209	1.07	5.39	6.40	588	22	1.68	84.48	0
OFF	Plains	1,434	4,528	234	2.56	3.56	4.11	317	17	3.23	141.78	3.24
HAR	Plains	1,459	975	139	4.49	3.19	13.72	193	16	0	207.95	4.88
SIM	Plains	1,487	371	122	5.22	7.85	16.03	266	15	0	286.95	3.72
NEW	Plains	1,511	1,387	165	5.59	1.47	12.83	328	16	3.07	73.60	0
NAM	Plains	1,524	238	98	8.17	0	12.22	21	8	0	143.95	47.77
GLA	Plains	1,544	262	97	0	0	3.66	21	10	0	385.73	0
LON	Plains	1,566	555	135	1.75	1.75	7.13	49	12	0	298.85	0
LIT	Foothills	1,598	64	57	0	0	0	7	4	0	359.92	0
NRW	Montane	1,688	7	7	0	0	0	0	0	0	0	0
VSP	Montane	1,737	46	45	0	0	0	0	0	0	0	0
IDY	Montane	1,840	4	4	0	0	0	0	0	0	0	0

Adult mosquitoes were tested in pools of 1–50. Bias-corrected maximum likelihood estimates for infection rates per 1,000 mosquitoes were calculated with Excel Add-In PooledInfRate 3.0.²⁴ LMP = Lee Martinez Park; BFH = Bellvue Fish Hatchery; PIR = Picnic Rock; GAP = Gateway Park; OYG = Ouzel; STP = Stove Prairie Landing; DUG = Dutch George; EGG = Eggers; DAD = Dadd Gulch; BSW = Bliss State Wildlife Area; OVE = Overland; ATW = Atwood State Wildlife Area; MES = Messex; COT = Cottonwood State Wildlife Area; JEA = Jean K. Tool State Wildlife Area; BOY = Boyd Ponds State Wildlife Area; WEL = Weldonia; SOO = South Orchard; GRE = Greeley; MIT = Mitani State Wildlife Area; BRO = Brower State Wildlife Area; OFF = Off CO60; HAR = Hardman; SIM = Simpson Ponds State Wildlife Area; NEW = Newell Lake; NAM = Namaqua Park; GLA = Glade Park; LON = Lonetree Reservoir; LIT = Little Dam; NRW = Narrows; VSP = Viestenz Smith Mountain Park; IDY = Idylwild.

* Based in part on data published previously by Barker and others.⁴⁷

between years are likely related, in part, to the fact that different sites were sampled in 2006 and 2007. The overall infection rates per 1,000 *Cx. pipiens* in 2006 were 0 for WNV, 462.42 for CxFV, and 0 for CLBOV. The corresponding infection rates for *Cx. pipiens* in 2007 were 2.41 for WNV, 72.09 for CxFV, and 3.18 for CLBOV. Notably, CxFV infection rates in *Cx. pipiens* were very high compared with the other two viruses.

In 2007, the WNV and CxFV infection rates in *Cx. tarsalis* increased gradually from June to September, whereas the CLBOV infection rates were highest in June (3.28) and then decreased slightly from July to September (Table 1 and Figure 2). WNV and CLBOV infection rates in *Cx. pipiens* followed similar seasonal patterns, with the highest infection rates occurring in June when mosquito counts were low (Table 1 and Figure 2).

Both insect-specific flaviviruses occurred widely in the five-county study area. CxFV was detected from all 21 sites in the plains sampled in 2006 and 2007, and CLBOV was recorded from 20 of these sites. The viruses also occurred in foothills sites but were not recorded from montane sites where mosquito counts are very low (Table 2). Site-specific infection

rates for WNV were compared with site-specific CxFV and CLBOV infection rates by graphing, but there were no apparent associations (data not shown).

Phylogenetic analyses. Bayesian analysis was conducted using a 1-kb region of the NS5 gene sequence to assess phylogenetic relationships between insect-specific flavivirus isolates from *Culex* spp. mosquitoes in Colorado and other selected flaviviruses (Figure 3). Neighbor-joining, maximum parsimony, and maximum likelihood analyses resulted in similar tree topologies (data not shown). The CxFV isolate from this study was most similar to CxFV isolates from Texas and was grouped with other CxFV isolates from Iowa, Japan, Mexico, and Guatemala (Figure 3). The Colorado CLBOV isolate shared the closest phylogenetic relationships with the CLBOV sequences detected in California and Alberta, Canada,^{32,33} encoding 97% NS5 amino acid sequence similarity.

Based on Bayesian analysis, the insect-specific flavivirus clade contains two subclades, which correspond with insect host genus. The first subclade contains CLBOV, Quang Binh virus, and the CxFV isolates, which have all been detected in *Culex* mosquitoes. The second subclade contains Kamiti

FIGURE 2. Monthly infection rates from June to September 2007 for *Cx. tarsalis* (A–C) and *Cx. pipiens* (D–F) with WNV (A and D), CxFV (B and E), and CLBOV (C and F). Error bars indicate 95% skewness-corrected confidence intervals. Estimates for infection rates per 1,000 mosquitoes were calculated with Excel Add-In PooledInfRate 3.0.²⁴

River virus, cell fusing agent virus, and *Aedes* flavivirus, which have all been described in *Aedes* mosquitoes. Interestingly, Nounané virus, which was recently isolated from *Uranotaenia* mosquitoes in Côte d'Ivoire,⁸ seems to only replicate in insect

cells and based on our phylogenetic analysis, groups with the arthropod-borne flaviviruses.

Virus isolation. Isolation of insect-specific flaviviruses from *Culex* in Colorado was attempted by blind passages in

FIGURE 3. Phylogenetic tree inferred from Bayesian analysis and midpoint rooted, showing relationships between insect-specific flaviviruses from *Culex* species mosquitoes collected in Colorado (shaded) and other flaviviruses based on a 1-kb segment of the NS5 gene. The numbers at the nodes represent clade credibility values and the scale bar indicates the number of estimated substitutions per site. GenBank accession numbers for the sequences used in the analysis are listed in parentheses.

Vero, DF-1, and C6/36 cell cultures. Vero (mammalian) and DF-1 (avian) cells inoculated with mosquito homogenate supernatants did not exhibit any CPE after one to four passages. RNA extractions were performed on Vero and DF-1 cell culture medium after each passage, and these tested negative by RT-PCR using virus-specific primers. In contrast, CxFV-infected *Cx. pipiens* homogenate supernatants caused minor growth inhibition for C6/36 (mosquito) cells after several passages. RNA extracted from cell culture medium was CxFV-positive by RT-PCR after each passage. CLBOV-infected *Cx. tarsalis* homogenate supernatants caused apparent CPE in C6/36 cells on 5 dpi only after 10 passages. RNA extractions of C6/36 cell culture medium for passages one to three were negative by RT-PCR using virus-specific primers, but after the fourth passage, they were positive. Spot slides of CxFV- and CLBOV-infected C6/36 cells were tested by IFA with JE, SLE, and WNV antibodies based on a previous report,¹⁴ but antigens were undetectable. IFA using virus-specific antibodies for CxFV and CLBOV produced positive results, confirming C6/36 cell infections.

CxFV infection in a *Cx. pipiens* laboratory colony. After detection of CxFV from adult mosquitoes collected in 2006 and 2007, our *Cx. pipiens* laboratory colony, established during the summer of 2005 from egg rafts collected in Fort Collins, was tested in March of 2007 and found to be infected with CxFV by RT-PCR and virus isolation. A subset of mosquitoes had been taken from the colony in September of 2005 and stored at -80°C . These stored specimens also tested positive for CxFV, indicating that the mosquitoes were infected at the time that the colony was established. Our initial RNA extractions were performed using a column-based method that is used for screening field-collected pools; however, we compared this with a Trizol extraction method for individual colony mosquitoes and found that Trizol extraction provided more sensitive detection. Briefly, males and females of various ages were aspirated from the colony, and RNA was extracted from individual mosquitoes using the column-based method and the Trizol method. Samples were tested by standard RT-PCR, with 1 of 28 mosquitoes testing positive for CxFV using the column-based RNA extraction method and 6 of 16 mosquitoes testing positive for CxFV using the Trizol RNA extraction method. To examine viral maintenance within this naturally infected colony, total RNA was extracted from individual egg rafts, individual fourth instar larvae, and individual adult (male and female) mosquitoes using Trizol and tested by a qRT-PCR assay with virus-specific primers. All life stages were found to be positive for CxFV RNA.

DISCUSSION

Culex mosquitoes collected in 2006–2007 in northeastern Colorado as part of ongoing studies to investigate entomological patterns of risk for exposure to WNV were found to be commonly infected with two insect-specific flaviviruses. Both viruses were isolated in C6/36 mosquito cells but failed to replicate in Vero mammalian cells or DF-1 avian cells. Phylogenetic analyses, based on a 1-kb sequence from the NS5 gene, revealed that these viruses group with previously described insect-specific flaviviruses. We isolated a strain of *Culex* flavivirus (CxFV) from *Cx. pipiens* and a novel insect-specific flavivirus, Calbertado virus (CLBOV), from *Cx. tarsalis*. A 1-kb sequence from the NS5 gene of the Colorado CLBOV isolate shares

> 90% nucleotide sequence identity and encodes 97% amino acid sequence identity with the CLBOV sequences identified from California and Alberta, Canada.^{32,33} The next most closely related viruses to CLBOV are CxFV isolates, with NS5 nucleotide sequences ranging from 65% to 68% similarity. After detecting these insect-specific flaviviruses in *Culex* from Colorado, we initiated studies to investigate seasonal and spatial patterns of infection, determine cell culture host range, and examine viral prevalence within a naturally infected laboratory colony.

Trends for insect-specific flaviviruses in *Culex* spp. in time and space. We found that infection rates for CxFV and CLBOV varied by *Culex* species, month, and site. In 2006, there was a strong species-specific pattern with CxFV being detected almost exclusively in *Cx. pipiens* and all records of CLBOV coming from *Cx. tarsalis*. In 2007, we collected and examined far greater numbers of *Culex* mosquitoes, and both insect-specific flaviviruses were detected in *Cx. tarsalis* as well as *Cx. pipiens*. These findings suggest that CxFV and CLBOV circulate in both species of mosquitoes in northern Colorado, although we cannot entirely rule out the possibility that a body part from one species sometimes was accidentally combined with a pool from the other species. *Culex* flavivirus has previously been detected in a variety of *Culex* spp., including *Cx. pipiens*,^{5,15} *Cx. tarsalis*,¹⁵ *Cx. quinquefasciatus* Say,^{5,12,14} *Cx. tritaeniorhynchus*,⁵ and *Cx. restuans* Theobald.¹⁴ These findings indicate that CxFV occurs in numerous *Culex* species. We also tested a subset of *Ae. vexans* and *Culiseta* spp. pools, but these were negative with the pan-flavivirus primers used. Other studies also found *Aedes* species pools to be negative for CxFV RNA.^{5,15}

We detected CLBOV RNA in both *Cx. tarsalis* and *Cx. pipiens* from Colorado and isolated the virus from several *Cx. tarsalis* pools. A portion of the NS5 gene of this virus shares a high similarity to viral sequences found in *Cx. tarsalis* mosquitoes in Alberta and other western provinces of Canada.^{32,33} Although a virus isolate has not yet been obtained from Alberta, detection of this sequence in a number of Canadian collections of *Culex* species suggests that CLBOV may have a wide geographic range. Field studies in California also indicate that CLBOV is present in *Cx. tarsalis* and *Cx. pipiens* populations there (Brault A, unpublished data).

Our data suggest that CxFV and CLBOV are prevalent in *Cx. tarsalis* and *Cx. pipiens* throughout the northeastern Colorado plains. In 2006, CxFV RNA was detected in April and every month from June to October (Table 1). In 2007, CxFV was detected in both species from June to September. In Texas, Kim and others¹⁴ recorded CxFV-positive mosquito pools during February and March, but continued surveillance from April to August resulted in only negative pools. In Iowa, CxFV RNA was detected from July to October but not in May or June, possibly because of low numbers of *Culex* collected early in the season.¹⁵

Our study yielded very high infection rates for CxFV in *Cx. pipiens*. Other studies have reported variable infection rates for CxFV, with minimum infection rates (MIR) per 1,000 *Cx. quinquefasciatus* ranging from 4.7 in Guatemala¹² to 20.8 in Mexico,¹³ and overall MIRs in Iowa ranging from 1.2 in *Cx. tarsalis* to 10.3 for *Cx. pipiens*.¹⁵ Both our study (Table 2) and the Iowa study recorded substantial site-specific variability for CxFV infection rates in a given species. Additional research is needed to determine the mechanisms resulting in spatial and temporal variability in CxFV infection rates.

We present the first data regarding seasonality of CLBOV, which were recorded in April 2006 and then every month from June to September in 2006 and 2007 (Table 1). Infection rates with CLBOV for months when at least 100 *Cx. tarsalis* or *Cx. pipiens* were examined ranged from 2.15 to 36.79. As a crude comparison, in Alberta, Canada, CLBOV was detected in 67 of 140 (48%) *Cx. tarsalis* mosquito pools tested from 2003 to 2005.^{32,33}

Comparison of seasonal infection rates for CxFV, CLBOV, and WNV. Sample sizes for *Culex* mosquitoes in 2007 were adequate to compare the seasonal (monthly) patterns for infection rates with CxFV, CLBOV, and WNV from June to September (Figure 2). This produced some unexpected and intriguing results. For instance, the infection rates in *Cx. tarsalis* for WNV and CxFV followed similar patterns, gradually increasing throughout the study period (Figure 2A and B), whereas the CLBOV infection rate showed an opposite, slightly decreasing trend from June to September (Figure 2C). The infection rates in *Cx. pipiens* with WNV, CxFV, and CLBOV followed the same general decreasing trend from June to September (Figure 2D–F), but the infection rates observed for CxFV were much higher compared with WNV and CLBOV. It is important to consider that high infection rates can be an artifact of low sample size, which is indicated by larger error bars when mosquito counts are low (Figure 2). We also recognize that the data for *Cx. pipiens* in our study should be interpreted with care, because use of CDC light traps can underestimate the abundance of this species compared with efforts that also include gravid traps.³⁴

Natural maintenance of CxFV and CLBOV. *Culex* flavivirus and CLBOV were detected in both male and female mosquito pools in our studies. Other studies investigating insect-specific flaviviruses have reported similar findings.^{5,7,11} Presence of insect-specific flaviviruses in mosquitoes of both sexes suggests vertical transmission as a mechanism of viral maintenance in nature.¹¹ Evidence supporting vertical transmission for KRV includes that the first isolates came from adult *Ae. macintoshi* mosquitoes that were collected as larvae and pupae from flooded dambos,⁴ and that female *Ae. aegypti*, infected with KRV by oral exposure, transmitted the virus to their offspring.³⁵ To investigate how insect-specific flaviviruses are maintained in mosquito populations, quantitative RT-PCR was performed on specimens from our laboratory colony of *Cx. pipiens* that was established from egg rafts collected in Fort Collins in 2005 and later found to be naturally and persistently infected with CxFV. Total RNA was extracted from individual egg rafts, single larvae, and single adults, with high proportions of all stages testing positive for CxFV. These results provide more evidence supporting vertical transmission as a means of viral maintenance in natural populations.

Vertical transmission seems to play a role in viral maintenance for other viruses in the genus *Flavivirus*. Vertical transmission of a flavivirus was first described in Senegal with isolation of Koutango virus from a male *Ae. aegypti*.³⁶ Since then, there have been numerous descriptions of flaviviruses isolated from larvae or male mosquitoes, including JEV from *Cx. tritaeniorhynchus*,³⁷ yellow fever from *Ae. aegypti*,³⁸ and WNV from *Cx. univittatus* Theobald,³⁹ and laboratory studies also have shown vertical transmission of flaviviruses.^{40–43} It is thought that flaviviruses are vertically transmitted at the time of oviposition during fertilization through the micro-pyle, as the fully developed egg passes through the oviduct.⁴⁴

This is much less efficient compared with true transovarial transmission, where the virus infects the developing egg.⁴⁵ Filial infection rates seen with vertically transmitted flaviviruses in mosquitoes are usually low (less than 1%) compared with much higher rates seen with bunyaviruses.⁴⁶ There are few data regarding the transmission dynamics of insect-specific flaviviruses, which have been found to replicate only in invertebrate cells. The inability to infect and replicate in vertebrate cells indicates that this group of flaviviruses has a distinct transmission cycle compared with the arthropod-borne flaviviruses, which are maintained between arthropod vectors and vertebrate hosts. Studies are planned to further investigate transmission dynamics of CxFV in our naturally infected *Cx. pipiens* colony.

If CxFV and CLBOV are indeed maintained in nature exclusively by vertical transmission, one might expect very high infection rates throughout the study area. This, however, was not observed by us or in other studies on CxFV. An important consideration with viral surveillance is the sensitivity of the assay used. Initial studies with the CxFV-infected laboratory colony after a column-based RNA extraction and standard RT-PCR resulted in detection of few CxFV-positive specimens. Although this method of RNA extraction was efficient and effective for mosquito pools, RNA extractions were performed using Trizol (Invitrogen) to increase sensitivity of RNA detection in individual mosquitoes from the laboratory colony, and this was followed by quantitative RT-PCR. These methods revealed a higher infection rate in the naturally infected colony. These observations suggest that viral titers in individual mosquitoes may sometimes be below the threshold of sensitivity for certain testing methods; however, we are confident that the column-based RNA extraction method combined with standard RT-PCR is adequate for detection of viral RNA in mosquito pools. It is unclear at this time why insect-specific flavivirus infection rates observed in this study and other studies are lower than would be expected if vertical transmission was an efficient means for viral maintenance in nature.

In conclusion, we detected two insect-specific flaviviruses in *Culex* spp. mosquitoes collected in northern Colorado: *Culex* flavivirus, first isolated in Japan,⁵ and Calbertado virus, a novel flavivirus. Based on 2 years of field studies, we have provided the first description of the seasonal dynamics of these viruses in Colorado. The common occurrence of these viruses in natural *Culex* populations raises questions regarding possible interactions with other flaviviruses that do cause disease in humans, such as WNV.³ Future studies are planned to determine how insect-specific flaviviruses may interact with arboviruses in a coinfecting mosquito and how this may potentially impact vector competence.

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Authors' addresses: Bethany G. Bolling, Division of Vector-Borne Diseases, Centers for Disease Control and Prevention, Fort Collins, CO, E-mail: bbolling@cdc.gov. Lars Eisen, Chester G. Moore, and Carol D. Blair, Arthropod-Borne and Infectious Diseases Laboratory, Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, CO, E-mails: lars.eisen@colostate.edu, chester.moore@colostate.edu, and carol.blair@colostate.edu.

Reprint requests: Bethany G. Bolling, Division of Vector-Borne Diseases, Centers for Disease Control and Prevention, 3150 Rampart Rd., Fort Collins, CO 80521, E-mail: bbolling@cdc.gov.

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