VECTOR-BORNE AND ZOONOTIC DISEASES
Volume 10, Number 8, 2010

DOI: 10.1089/vbz.2009.0196

Detection of Flaviviruses and Orthobunyaviruses in Mosquitoes in the Yucatan Peninsula of Mexico in 2008

Jose A. Farfan-Ale,[1] Maria A. Loroño-Pino,[1] Julian E. Garcia-Rejon,[1] Victor Soto,[2] Ming Lin,[2] Molly Staley,[2] Karin S. Dorman,[3] Lyric C. Bartholomay,[4] Einat Hovav,[2] and Bradley J. Blitvich[2]

Abstract

A total of 191,244 mosquitoes from 24 species were collected in the Yucatan Peninsula of Mexico from January to December 2008, and tested for the presence of cytopathic virus by virus isolation in Vero cells. Eighteen virus isolates were obtained, all of which were orthobunyaviruses. These were identified by reverse transcription-polymerase chain reaction (RT-PCR) and nucleotide sequencing as Cache Valley virus ($n = 17$) and South River virus ($n = 1$). A subset ($n = 20{,}124$) of *Culex quinquefasciatus* collected throughout the year was further tested by RT-PCR using flavivirus-specific primers. Flavivirus RNA was present in this mosquito species year-round. The overall flavivirus minimal infection rate, expressed as the number of positive mosquito pools per 1000 mosquitoes tested, was 7.7 and the monthly flavivirus minimal infection rates ranged from 4.3 to 16.6. Approximately one-third of the RT-PCR products were sequenced and all corresponded to Culex flavivirus, a recently discovered insect-specific flavivirus.

Key Words: Bunyavirus—Cache Valley virus—Culex flavivirus—*Flavivirus*—Mexico—Mosquito—South River virus—Surveillance.

Introduction

Mosquito-transmitted viruses have an enormous impact on human and animal health throughout the world, especially in the tropics and subtropics, where the climate is conducive for year-round mosquito proliferation. Mosquito-transmitted viruses of greatest medical and veterinary significance belong primarily to three genera: *Flavivirus* (family Flaviviridae), *Alphavirus* (family Togaviridae), and *Orthobunyavirus* (family Bunyaviridae). In Latin America, mosquito-borne viruses associated with human illness include Dengue, Yellow fever, West Nile, St. Louis encephalitis and Ilheus viruses (all of which belong to the genus *Flavivirus*), Venezuelan equine encephalitis, Eastern equine encephalitis, Western equine encephalitis and Mayaro viruses (*Alphavirus*), and Oropouche and Fort Sherman viruses (*Orthobunyavirus*) (Weaver 2005, Griffin 2007, Gubler et al. 2007, Schmaljohn and Nichol 2007, Petersen and Hayes 2008).

Comprehensive surveillance for West Nile virus (WNV) and other mosquito-transmitted viruses has been performed in the Yucatan Peninsula of Mexico since 2001 (Farfan-Ale et al. 2004, Farfan-Ale et al. 2006, 2009, Lorono-Pino et al. 2003, 2004, 2009). Recently, we reported data from entomologic studies performed in the states of Yucatan and Quintana Roo from January to December 2007 (Farfan-Ale et al. 2009). In this study, 96,687 mosquitoes were collected and assayed by virus isolation in mammalian cells. Three isolates were obtained: one was Cache Valley virus (CVV; *Orthobunyavirus*), another was Kairi virus (*Orthobunyavirus*), and the identity of the third was not determined. A subset of mosquitoes (210 pools of *Culex quinquefasciatus*) was further tested by reverse transcription-polymerase chain reaction (RT-PCR) using flavivirus-specific primers. Of these, 145 (69%) pools contained RNA to Culex flavivirus (CxFV), an insect-specific virus first isolated from *Culex* spp. mosquitoes in Japan and Indonesia (Hoshino et al. 2007), and later from *Culex* spp. mosquitoes in Guatemala, Mexico, Trinidad, the United States, and Uganda (Morales-Betoulle et al. 2008, Blitvich et al. 2009, Cook et al. 2009, Farfan-Ale et al. 2009, Kim et al. 2009). Another pool contained RNA to a novel flavivirus (designated T'Ho virus)

[1]Laboratorio de Arbovirologia, Centro de Investigaciones Regionales Dr. Hideyo Noguchi, Universidad Autonoma de Yucatan, Merida, Mexico.
[2]Department of Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa.
Departments of [3]Genetics, Development, and Cell Biology and [4]Entomology, College of Agriculture and Life Sciences, Iowa State University, Ames, Iowa.

and that is genetically equidistant to several other flaviviruses including WNV (Farfan-Ale et al. 2009).

Here, we report the findings from our mosquito-based virus surveillance performed in the Yucatan Peninsula of Mexico from January to December 2008. The purpose of this study was to increase our knowledge of the biodiversity and distribution of the different species of mosquitoes in the Yucatan Peninsula of Mexico, to detect and isolate new and previously described arboviruses from different mosquito species, and to genetically and phylogenetically characterize these viruses.

Materials and Methods

Description of study sites

Study sites were located in five areas: Merida (urban), Tixkokob (semi-urban), and Celestun (semi-urban) in Yucatan State, and Sian Ka'an (rural) and Isla Mujeres (semi-urban) in Quintana Roo State. The study area in Merida covered approximately 40 km^2; all other study areas were less than 4 km^2. There were 15 study sites located in Merida, and 5 of these were in the Parque del Centenario Zoologico (the Merida zoo). The Global Positioning System (GPS) locations of the study sites in Merida range from 20°59′25″ N to 20°55′32″ N, and 89°35′42″ W to 89°38′22″ W. Two study sites were located in Tixkokob (GPS location 20°59′38″ N and 89°23′14″ W), an area used for horse and cattle production. In Celestun (GPS location 20°51′7″ N and 90°23′24″ W), eight study sites were established. Celestun is a small coastal town and many species of birds overwinter in this area. Eleven study sites were located on Sian Ka'an (GPS location 20°17′54″ N and 87°15′22″ W), a small island 3 km off the east coast of the Mexican mainland. The island forms part of the Sian Ka'an Biosphere Reserve. In Isla Mujeres (GPS location 21°12′51″ N and 86°43′24″ W), seven study sites were established. Isla Mujeres is a small island town 7 km off the east coast of the Mexican mainland. All study areas used for the mosquito collections have a tropical climate.

Sampling methods and trapping schedules

Mosquitoes were sampled using Mosquito Magnet™ (American Biophysics Corp., North kingstown, RI) traps and Centers for Disease Control and Prevention backpack-mounted aspirators as previously described (Farfan-Ale et al. 2009). In Merida, collections were made from January to December 2008. Each site was trapped 5 days a week every week. One mosquito magnet and/or two backpack-mounted aspirators were used at each site. Tixkokob was visited from January to March. Every site was trapped 5 days a week every other week using one mosquito magnet or two backpack-mounted aspirators. The other locations were visited once (Celestun from December 2–5; Isla Mujeres from November 10–14, and Sian Ka'an from December 16–19). Each site was trapped daily using one mosquito magnet. Mosquitoes from Yucatan State were transported live to the Universidad Autonoma de Yucatan (UADY), frozen at −70°C, and then identified on chill tables according to species and sex using morphological characteristics. Mosquitoes from Quintana Roo State were anesthetized using triethylamine (Kramer et al. 1990), identified, placed into cryostorage vials, and transported in liquid nitrogen to the UADY. Mosquitoes were transported on dry ice from the UADY to Iowa State University.

Mosquito homogenization and virus isolation in Vero cells

Mosquitoes were homogenized in CO_2-independent cell culture medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and various other supplements as previously described (Farfan-Ale et al. 2009). Homogenates were centrifuged (10,000 *g*, 10 min, 4°C), and supernatants were collected. An aliquot of each supernatant was tested for the presence of cytopathic virus by virus isolation in African Green Monkey Kidney (Vero) cells as previously described (Farfan-Ale et al. 2009).

Virus identification by RT-PCR sequencing

Total RNA was extracted from cell culture supernatants and mosquito homogenates using the QIAamp viral RNA extraction kit (Qiagen, Valencia, CA) and analyzed by RT-PCR using primers specific for flaviviruses, orthobunyaviruses, and alphaviruses. The flavivirus-specific primers, FU2 and cFD3, target a 845-nt region of the *NS5* gene (Kuno et al. 1998). The orthobunyavirus-specific primers, BCS82C and BCS332V, target a 251-nt region of the small (S) RNA segment (Kuno et al. 1996). The alphavirus-specific primers, VIR966 and VIR966c, target a 98-nt region of the *nsP1* gene (Eshoo et al. 2007). Previously described primers specific for WNV (Lanciotti et al. 2000) and T'Ho virus (Farfan-Ale et al. 2009) were also used. Complementary DNA was generated using Superscript III reverse transcriptase (Invitrogen) and PCRs were performed using *Taq* polymerase (Invitrogen). RT-PCR products were purified using the Purelink Gel Extraction Kit (Invitrogen) and sequenced using a 3730x1 DNA sequencer (Applied Biosystems, Foster City, CA).

Results

Mosquito collections

A total of 191,244 mosquitoes were collected in the Yucatan Peninsula of Mexico from January to December 2008 (Table 1). Of these, 151,851 (79%) were identified as females and 39,393 (21%) were identified as males. The mosquitoes represent 11 genera and 24 species. The most common species was *Ochlerotatus* (*Aedes*) *taeniorhynchus*, which made up 59% of the total sample population, followed by *Cx. quinquefasciatus* (34%). The majority (82%) of mosquitoes was collected using Mosquito Magnet traps; the remainder (18%) was collected by manual aspiration (data not shown).

Collections were made at multiple sites in five study areas: Celestun, Merida, and Tixkokob in Yucatan State, and Sian Ka'an and Isla Mujeres in Quintana Roo State (Table 2). Most time and effort was devoted to the collection of mosquitoes in Merida, particularly the Merida zoo, because WNV and T'Ho virus activity had previously been detected in this area (Farfan-Ale et al. 2006, 2009). A total of 53,410 mosquitoes were collected in the Merida zoo and another 96,901 mosquitoes were collected at other sites in Merida. Together, these mosquitoes made up 79% of the total sample population. The collections from the Merida zoo consisted of nine species, and the most common species was *Cx. quinquefasciatus*; it represented 85% of the mosquitoes collected in this site. Sixteen

Table 1. Summary of Mosquitoes Collected in the Yucatan Peninsula of Mexico, January–December 2008

Species	Number of pools	Number of mosquitoes			% Total sample population
		Female	Male	Total	
Aedes aegypti	701	2024	3861	5885	3.1
Anopheles albimanus	43	551	0	551	0.3
An. crucians	60	1029	0	1029	0.6
An. vestitipennis	63	1427	0	1427	0.7
Coquillettidia nigricans	7	62	0	62	<0.1%
Cq. venezuelensis	2	17	0	17	<0.1%
Culex bahamensis	3	3	0	3	<0.1%
Cx. coronator	19	154	0	154	0.1
Cx. interrogator	46	716	50	766	0.4
Cx. nigripalpus	14	235	0	235	0.1
Cx. opisthopus	39	697	1	698	0.4
Cx. quinquefasciatus	2080	30,902	35,026	65,928	34.5
Deinocerites cancer	2	6	0	6	<0.1%
Haemagogus mesodentatus	1	1	0	1	<0.1%
Mansonia titillans	11	19	0	19	<0.1%
Ochlerotatus (*Aedes*) *fulvus pallens*	3	5	0	5	<0.1%
Oc. (*Aedes*) *sollicitans*	1	1	0	1	<0.1%
Oc. (*Aedes*) *taeniorhynchus*	2841	113,107	455	113,562	59.4
Oc. (*Aedes*) *trivittatus*	37	600	0	600	0.3
Psorophora cyanescens	10	155	0	155	0.1
Ps. ferox	10	25	0	25	<0.1%
Ps. howardii	5	29	0	29	<0.1%
Uranotaenia lowii	6	27	0	27	<0.1%
Wyeomyia mitchellii	5	59	0	59	<0.1%
Total	6009	151,851	39,393	191,244	100.0

Table 2. Numbers of Mosquitoes Collected According to Study Area

Species	Study area						Total
	Celestun	Isla Mujeres	Merida[a]	Sian Ka'an	Tixkokob	Zoo	
Ae. aegypti	0	105	301	0	475	5004	5885
An. albimanus	35	0	274	239	1	2	551
An. crucians	376	34	3	616	0	0	1029
An. vestitipennis	0	0	85	1337	4	1	1427
Cq. nigricans	0	0	0	62	0	0	62
Cq. venezuelensis	0	0	0	17	0	0	17
Cx. bahamensis	0	0	0	3	0	0	3
Cx. coronator	0	0	150	0	0	4	154
Cx. interrogator	0	24	646	3	3	90	766
Cx. nigripalpus	0	213	19	0	0	3	235
Cx. opisthopus	0	0	0	698	0	0	698
Cx. quinquefasciatus	331	3669	3630	10	12,866	45,422	65,928
D. cancer	0	0	0	6	0	0	6
H. mesodentatus	0	0	1	0	0	0	1
Mn. titillans	0	0	18	1	0	0	19
Oc. (*Aedes*) *fulvus pallens*	0	0	2	3	0	0	5
Oc. (*Aedes*) *sollicitans*	0	0	0	1	0	0	1
Oc. (*Aedes*) *taeniorhynchus*	17,337	1628	91,374	114	234	2875	113,562
Oc. (*Aedes*) *trivittatus*	1	5	236	346	3	9	600
Ps. cyanescens	0	0	153	2	0	0	155
Ps. ferox	1	0	0	24	0	0	25
Ps. howardii	0	0	5	24	0	0	29
U. lowii	0	0	4	23	0	0	27
W. mitchellii	0	0	0	59	0	0	59
Total	18,081 (9.4%)	5678 (3.0%)	96,901 (50.7%)	3588 (1.9%)	13,586 (7.1%)	53,410 (27.9%)	191,244 (100%)

[a]Does not include mosquitoes collected in the Merida zoo.

species were collected elsewhere in Merida, and the most common (94%) species was *Oc. taeniorhynchus*. Six species were collected in Celestun, and the majority (96%) were *Oc. taeniorhynchus*. In Tixkokob, seven species were collected, and the most common (95%) was *Cx. quinquefasciatus*. Species diversity was greatest in Sian Ka'an; 20 species were collected in this area, and the most common (37%) was *Anopheles vestitipennis*. Eight species were collected in the Isla Mujeres, and *Cx. quinquefasciatus* was the most common (65%).

Virus isolations in Vero cells

Eighteen of 6009 mosquito pools caused virus-like cytopathic effect (CPE) in Vero cells. All 18 pools consisted of female *Oc. taeniorhynchus* collected at four study sites in Celestun in December 2008. The sites are within 9 km (6 miles) of each other. Collections were made using Mosquito Magnet traps (13 pools) and backpack-mounted aspirators (5 pools). Seventeen isolates were identified by RT-PCR sequencing as CVV (GenBank accession numbers GU018033–GU018049), and the other as South River virus (SORV; GenBank accession number GU018050). The CVV and SORV minimal infection rates (MIRs) in *Oc. taeniorhynchus* in Celestun were 0.98 and 0.06, respectively.

Sequence analysis of the CVV small genomic RNA segment

The complete S RNA genome segments of five CVV isolates from Celestun were sequenced, with the exception of an estimated 22-nt at both the 5′ and 3′ termini, using three pairs of overlapping primers (primer sequences are available upon request). As with other members of the genus *Orthobunyavirus*, the S RNA segment of CVV encodes for the nucleocapsid and NS_S proteins in overlapping reading frames (Dunn et al. 1994, Schmaljohn and Nichol 2007). Additional sequencing was also performed on a CVV isolate collected in Merida in 2007 (designated CVV-Mex07) (Farfan-Ale et al. 2009). The nucleotide sequences of the S RNA segments of the five isolates from Celestun were aligned using the CLUSTAL W algorithm (version 2) (Higgins and Sharp 1988, Larkin et al. 2007) and shown to be 98.7% to 100% identical. All but 12 of the 906 nucleotide positions were strictly conserved between all of these isolates. Pairwise alignments of these sequences to the homologous region of the prototype CVV strain 6V633 (GenBank accession number X73465), which is the only CVV isolate for which complete S RNA genome sequence data are available, revealed 91.1% to 97.8% identity. The deduced amino acid sequences of the nucleocapsid proteins of the five CVV isolates from Celestun are 100% identical to one another, as are the deduced amino acid sequences of the NS_S proteins. Pairwise alignments of the deduced amino acid sequences of the nucleocapsid and NS_S proteins of each isolate to that of the prototype strain revealed 99.1% and 100% identity, respectively, in all cases.

Phylogenetic analysis of the CVV small genomic RNA segment

A phylogenetic tree was constructed with Bayesian methods using a 795-nt region of the S RNA segments of the 5 CVV isolates from Celestun and 19 other orthobunyaviruses from the Bunyamwera serogroup (Fig. 1). Three other CVV isolates are included in this analysis: the prototype strain, CVV-Mex07, and an isolate from Wisconsin in 2003 (designated strain WI-03BS7669); these are the only other CVV isolates that have had most, if not all, of their S RNA segments sequenced. Phylogenetic trees were also generated using neighbor-joining, maximum parsimony, and maximum likelihood methods. In the Bayesian tree, the Celestun isolates of

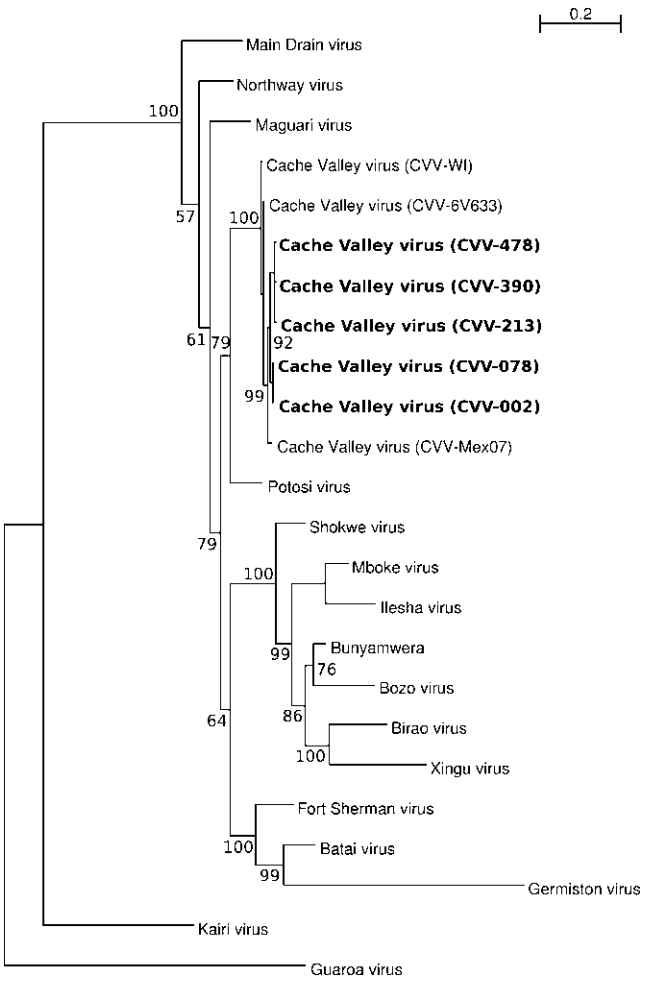

FIG. 1. Phylogenetic analysis of a 795 nucleotide region of the small RNA genome segment of 5 CVV isolates obtained in this study and 19 other bunyavirus isolates. The displayed phylogeny was estimated by the program MRBAYES, version 3.1 (Ronquist and Huelsenbeck 2003). Posterior support (out of 100) for selected branches is indicated. An unrooted tree was inferred but is shown rooted using the midpoint method. CVV isolates obtained in the present study are denoted in bold. GenBank accession numbers for sequences used in the phylogenetic analysis are as follows: Batai virus (X73464.1), Birao virus (AM711131.1), Bozo virus (AM711132.1), Bunyamwera virus (D00353.1), CVV strain 6V633 (X73465), CVV strain CVV-002 (GU018033), CVV strain CVV-078 (GU018034), CVV strain CVV-213 (GU018035), CVV strain CVV-390 (GU018036), CVV strain CVV-478 (GU018037), CVV strain CVV-Mex07 (EU879062), CVV strain WI-03BS7669 (DQ315775), Fort Sherman virus (EU564829.1), Germiston virus (M19420.1), Guaroa virus (X73466.1), Ilesha virus (AM709780.1), Kairi virus (EU879063), Maguari virus (M28380.1), Main Drain virus (X73469.1), Mboke virus (AY593727.1), Northway virus (X73470.1), Potosi virus (AY729652.1), Shokwe virus (EU564831.1), and Xingu virus (EU564830.1). CVV, Cache Valley virus.

CVV share a close phylogenetic relationship with each other and with the other CVV isolates; the bootstrap supports for these topological arrangements are 92% and 100%, respectively (Fig. 1). Phylogenetically, the S segments of the CVV isolates are most closely related to the homologous region of Potosi virus. The bootstrap support for this topological arrangement is 79%. The topologic arrangements of the neighbor-joining, maximum parsimony, and maximum likelihood trees were similar to the Bayesian tree (data not shown).

Detection of CxFV RNA in mosquito homogenates

A subset of mosquito homogenates was directly tested by RT-PCR using flavivirus-specific primers. Overall, 839 pools consisting of 23,561 mosquitoes from 8 species were tested: *Aedes aegypti* ($n = 638$; 52 pools), *Anopheles albimanus* ($n = 474$; 39 pools), *An. vestitipennis* ($n = 379$; 19 pools), *Cx. quinquefasciatus* ($n = 20{,}124$; 600 pools), *Ochlerotatus trivittatus* ($n = 1294$; 50 pools), *Oc. taeniorhynchus* ($n = 408$; 36 pools), *Mansonia titillans* ($n = 89$; 33 pools), and *Psorophora cyanescens* ($n = 155$; 10 pools). The *Cx. quinquefasciatus* used for this study were collected throughout the year (50 pools per month); all were from the Merida zoo, and approximately equal numbers of females and males were used. *Aedes*, *Anopheles*, *Mansonia*, *Ochlerotatus*, and *Psorophora* spp. mosquitoes were collected primarily from August to December; all were from Merida and Sian Ka'an, and most were female. Some of the *Mn. titillans* used in this analysis were collected in 2007 (Farfan-Ale et al. 2009).

Flavivirus RNA was detected in 155 (26%) pools of *Cx. quinquefasciatus* (Table 3) but in none of the *Aedes*, *Anopheles*, *Mansonia*, *Ochlerotatus*, and *Psorophora* spp. mosquitoes. *Cx. quinquefasciatus* with flavivirus RNA were collected each month. The overall flavivirus MIR in *Cx. quinquefasciatus* was 7.7. The highest monthly flavivirus MIRs occurred in December and August (16.6 and 12.4, respectively), and the lowest monthly flavivirus MIRs occurred in May to July (4.3–4.6). The annual flavivirus MIRs in female and male *Cx. quinquefasciatus* were similar (8.3 and 7.2, respectively; data not shown). A subset ($n = 50$) of RT-PCR products were sequenced using the flavivirus-specific forward primer, and all are >96% identical to the homologous region of CxFV-Mex07, the prototype Yucatan strain of CxFV (Farfan-Ale et al. 2009). Weak bands of approximately 800 nt were observed after the RT-PCR amplification and gel electrophoresis analysis of 19 *Oc. trivittatus* samples, but nucleotide sequencing revealed that these RT-PCR products were derived from the mosquito 28S ribosomal gene. One of these sequences has been entered into the GenBank database (GenBank accession number GU018051). Lastly, all of the female *Cx. quinquefasciatus* collected in the Merida zoo from June to December were tested by RT-PCR using a pooled suspension of T'Ho and WNV-specific primers; all were negative (data not shown).

Table 3. Seasonal Prevalence of Flaviviruses in *Culex quinquefasciatus* in the Yucatan Peninsula of Mexico

Month	Number of mosquitoes tested	Number of pools with flavivirus RNA[a]	Flavivirus MIR
January	2020	10	5.0
February	2147	10	4.7
March	1451	13	9.0
April	1851	22	11.9
May	1937	9	4.6
June	2272	10	4.4
July	2093	9	4.3
August	1537	19	12.4
September	846	9	10.6
October	1133	6	5.3
November	1206	11	9.1
December	1631	27	16.6
Total	20,124	155	7.7

[a]A total of 600 pools (50 per month) were tested.

Discussion

Entomologic surveillance for mosquito-transmitted viruses was conducted in the Yucatan Peninsula of Mexico from January to December 2008. A total of 191,244 mosquitoes in 6009 pools were tested for cytopathic virus by virus isolation in mammalian cells. Eighteen isolates were obtained; 17 were identified as CVV and the other as SORV. CVV is an orthobunyavirus in the Bunyamwera serogroup that is distributed across Canada, Mexico, and the United States (Calisher et al. 1986, Aguirre et al. 1992, Schmaljohn and Nichol 2007, Pabbaraju et al. 2009). CVV has been responsible for two documented cases of human disease, both of which occurred in the United States, as well as disease and pregnancy loss in domestic ruminants (Sexton et al. 1997, de la Concha-Bermejillo 2003, Campbell et al. 2006). Because CVV was isolated from mosquitoes in Merida in 2007 (Farfan-Ale et al. 2009) and Celestun in 2008 (this study), we speculate that this virus is common in the Yucatan Peninsula of Mexico. There is limited genetic and phylogenetic diversity between CVV isolates from Mexico and other geographical locations. SORV is an orthobunyavirus in the California serogroup that was first isolated in New Jersey in 1960 (Sudia et al. 1971) and later in Pennsylvania (Wills et al. 1974) and Georgia (D.G. Mead, pers. comm.). It is not known whether SORV is a pathogen of humans or animals. This is the first report of SORV in Latin America.

One virus was isolated for every 10,625 mosquitoes tested, and thus the proportion of mosquitoes containing cytopathic virus was low. Similar findings were observed in our entomologic investigations in the Yucatan Peninsula of Mexico in 2007 (1 virus per 32,229 mosquitoes) (Farfan-Ale et al. 2009). It is surprising that WNV was not detected in any of these collections, especially those from Merida, where trapping was performed year-round and where serologic studies have provided evidence of previous WNV transmission (Farfan-Ale et al. 2004, 2006, Lorono-Pino et al. 2003, 2009). These findings could indicate that WNV is maintained in the Yucatan Peninsula of Mexico in temporary, dynamic foci. These findings could also be due to a decrease in the intensity of WNV transmission in the Yucatan Peninsula of Mexico since the introduction of this virus into the region. For example, a fourfold decrease in WNV infection rates occurred in *Culex tarsalis* in a high transmission zone in Colorado from 2003 (MIR 34.5) to 2004 (MIR 8.7) (Bolling et al. 2007). A small number of WNV isolates have been obtained from Latin America and the Caribbean relative to the number of isolations made in the United States. The only regions in Latin

America and the Caribbean from where multiple isolations of WNV have been made are Puerto Rico (Barrera et al. 2008) and Guatemala (M.E. Morales-Betoulle, pers. comm.). In both studies, sentinel chicken surveillance was used to monitor ongoing or recent WNV transmission, and mosquito trapping was immediately initiated at sites where seroconversions were identified. Thus, sentinel chicken surveillance followed by targeted entomological surveillance should accompany ongoing mosquito-based surveillance in regions where WNV transmission is not accompanied by widespread human, equine, and avian illness to increase the likelihood of obtaining WNV isolates. However, it is important to note that the inclusion of sentinel chicken surveillance should not detract from the importance of performing ongoing mosquito-based surveillance because the latter is widely regarded to be the primary tool for quantifying the intensity of WNV transmission in an area (Centers for Disease Control and Prevention 2003). T'Ho virus was not detected in any mosquitoes collected in this study despite the considerable amount of time and effort devoted to the collection of mosquitoes in and around the Merida zoo. This finding indicates that T'Ho virus may also be maintained in the Yucatan Peninsula of Mexico in temporary, dynamic foci or that there are notable fluctuations in the intensity of T'Ho virus transmission in this region each year. It is not known whether sentinel chicken surveillance followed by targeted entomological surveillance provides a suitable approach to detect T'Ho virus because the vertebrate hosts of this virus have not been identified.

The flavivirus MIR in *Cx. quinquefasciatus* collected in the Merida zoo from January to December was 7.7. Nucleotide sequencing data indicate that most (if not all) of these mosquito homogenates contain CxFV RNA. The CxFV sequences were not entered into the GenBank database because the sequencing reactions were performed in one direction using a single primer (FU2). However, a comprehensive sequencing study is currently being performed in our laboratories using CxFV isolates from Mexico. The CxFV MIR (10.9) in *Cx. quinquefasciatus* in the Merida zoo from June to August 2007 was also high (Farfan-Ale et al. 2009). The presence of CxFV RNA in *Cx. quinquefasciatus* collected each month suggests that this virus is active in the Yucatan Peninsula of Mexico year-round. In contrast, CxFV was detected in *Culex pipiens* in Iowa from July through October, but not in any mosquitoes collected in May or June (Blitvich et al. 2009). In Texas, CxFV was identified in *Cx. quinquefasciatus* and/or *Culex restuans* from November to March, but in none of the mosquitoes collected in April to August (Kim et al. 2009). These data could indicate that CxFV activity is seasonal in certain areas or that persistence varies between different *Culex* spp. mosquitoes. The variations in monthly flavivirus MIRs (4.3–16.6) in *Cx. quinquefasciatus* in Merida may be due to the reasonably small number of mosquitoes tested (50 pools per month). The identification of CxFV RNA in approximately equal numbers of male and female mosquitoes suggests that CxFV is maintained in nature by vertical transmission.

CxFV was not detected in any *Aedes*, *Anopheles*, *Mansonia*, *Ochlerotatus*, and *Psorophora* spp. mosquitoes. These findings were not unexpected since all previous isolations of CxFV have been made from *Culex* spp. mosquitoes (*Cx. pipiens*, *Cx. quinquefasciatus*, *Cx. restuans*, *Cx. tarsalis*, and *Cx. tritaeniorhynchus*) (Hoshino et al. 2007, Morales-Betoulle et al. 2008, Blitvich et al. 2009, Cook et al. 2009, Farfan-Ale et al. 2009, Kim et al. 2009). However, other insect-specific flaviviruses have been identified in non-*Culex* mosquitoes. For instance, Kamiti River virus was isolated from *Aedes macintoshi* collected in Kenya in 1999 (Sang et al. 2003, Cook et al. 2006). More recently, Aedes flavivirus was detected in *Aedes albopictus* and *Aedes flavopictus* in Japan (Hoshino et al. 2009), and Nakiwogo virus was detected in *Mansonia africana nigerrima* in Uganda (Cook et al. 2009). This study provides no evidence that insect-specific flaviviruses, other than CxFV, are present in Mexico although it is important to note the small numbers of non-*Culex* spp. mosquitoes tested by RT-PCR for flavivirus RNA.

In summary, we report the detection of bunyaviruses (CVV and SORV) and flaviviruses (CxFV) in mosquitoes in the Yucatan Peninsula of Mexico. Due to the enormous impact that arboviruses have on human and animal health and the sparse number of comprehensive studies that have explored the diversity of viruses in mosquito populations in Mexico, additional entomologic investigations are required in this region.

Acknowledgments

The authors thank the field workers from the UADY. These individuals are as follows: Carlos Baak, Mildred López, Carlos Estrella, Alex Ic, Roger Arana, Wilberth Chi, Hugo Valenzuela, Iván Villanueva, Jesús Miss, Rosa Cetina, Lourdes Talavera, and Roger López. This study was supported by grant 5R21AI067281-02 from the National Institutes of Health.

Disclosure Statement

No completing financial interests exist.

References

Aguirre, AA, McLean, RG, Cook, RS, Quan, TJ. Serologic survey for selected arboviruses and other potential pathogens in wildlife from Mexico. J Wildl Dis 1992; 28:435–442.

Barrera, R, Hunsperger, E, Munoz-Jordan, JL, Amador, M, et al. First isolation of West Nile virus in the Caribbean. Am J Trop Med Hyg 2008; 78:666–668.

Blitvich, BJ, Lin, M, Dorman, KS, Soto, V, et al. Genomic sequence and phylogenetic analysis of Culex flavivirus, an insect-specific flavivirus, isolated from *Culex pipiens* (Diptera: Culicidae) in Iowa. J Med Entomol 2009; 46:934–941.

Bolling, BG, Moore, CG, Anderson, SL, Blair, CD, Beaty, BJ. Entomological studies along the Colorado front range during a period of intense West Nile virus activity. J Am Mosq Control Assoc 2007; 23:37–46.

Calisher, CH, Francy, DB, Smith, GC, Muth, DJ, et al. Distribution of Bunyamwera serogroup viruses in North America, 1956–1984. Am J Trop Med Hyg 1986; 35:429–443.

Campbell, GL, Mataczynski, JD, Reisdorf, ES, Powell, JW, et al. Second human case of Cache Valley virus disease. Emerg Infect Dis 2006; 12:854–856.

Centers for Disease Control and Prevention. Epidemic/Epizootic West Nile Virus in the United States: Guidelines for Surveillance, Prevention, and Control. www.cdc.gov/ncidod/dvbid/westnile/resources/wnv-guidelines-aug-2003.pdf, 2003.

Cook, S, Bennett, SN, Holmes, EC, De Chesse, R, et al. Isolation of a new strain of the flavivirus cell fusing agent virus in a natural mosquito population from Puerto Rico. J Gen Virol 2006; 87(Pt 4):735–748.

Cook, S, Moureau, G, Harbach, R, Mukwaya, L, et al. Isolation of a new species of flavivirus and a novel strain of Culex flavi-

virus (Flaviviridae), from a natural mosquito population in Uganda. J Gen Virol 2009:2664–2678.

de la Concha-Bermejillo, A. Cache Valley virus is a cause of fetal malformation and pregnancy loss in sheep. Small Ruminant Res 2003; 49:1–9.

Dunn, EF, Pritlove, DC, Elliott, RM. The S RNA genome segments of Batai, Cache Valley, Guaroa, Kairi, Lumbo, Main Drain and Northway bunyaviruses: sequence determination and analysis. J Gen Virol 1994; 75(Pt 3):597–608.

Eshoo, MW, Whitehouse, CA, Zoll, ST, Massire, C, et al. Direct broad-range detection of alphaviruses in mosquito extracts. Virology 2007; 368:286–295.

Farfan-Ale, JA, Blitvich, BJ, Lorono-Pino, MA, Marlenee, NL, et al. Longitudinal studies of West Nile virus infection in avians, Yucatan State, Mexico. Vector Borne Zoonot Dis 2004; 4:3–14.

Farfan-Ale, JA, Blitvich, BJ, Marlenee, NL, Lorono-Pino, MA, et al. Antibodies to West Nile virus in asymptomatic mammals, birds, and reptiles in the Yucatan Peninsula of Mexico. Am J Trop Med Hyg 2006; 74:908–914.

Farfan-Ale, JA, Lorono-Pino, MA, Garcia-Rejon, JE, Hovav, E, et al. Detection of RNA from a Novel West Nile-like virus and high prevalence of an insect-specific flavivirus in mosquitoes in the Yucatan Peninsula of Mexico. Am J Trop Med Hyg 2009; 80:85–95.

Griffin, DE. Alphaviruses. In: Knipe, DM, ed. *Fields Virology*, fifth edition. Philadelphia: Lippincott Williams and Wilkins, 2007:1023–1067.

Gubler, DJ, Kuno, G, Markoff, L. Flaviviruses. In: Knipe, DM, Howley, PM, eds. *Fields Virology*, fifth edition. Philadelphia, PA: Lippincott Williams and Wilkins, 2007:1153–1252.

Higgins, DG, Sharp, PM. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene 1988; 73:237–244.

Hoshino, K, Isawa, H, Tsuda, Y, Sawabe, K, Kobayashi, M. Isolation and characterization of a new insect flavivirus from *Aedes albopictus* and *Aedes flavopictus* mosquitoes in Japan. Virology 2009; 391:119–129.

Hoshino, K, Isawa, H, Tsuda, Y, Yano, K, et al. Genetic characterization of a new insect flavivirus isolated from *Culex pipiens* mosquito in Japan. Virology 2007; 359:405–414.

Kim, DY, Guzman, H, Bueno, R, Jr., Dennett, JA, et al. Characterization of Culex flavivirus (Flaviviridae) strains isolated from mosquitoes in the United States and Trinidad. Virology 2009; 386:154–159.

Kramer, LD, Presser, SB, Houk, EJ, Hardy, JL. Effect of the anesthetizing agent triethylamine on western equine encephalomyelitis and St. Louis encephalitis viral titers in mosquitoes (Diptera: Culicidae). J Med Entomol 1990; 27:1008–1010.

Kuno, G, Chang, GJ, Tsuchiya, KR, Karabatsos, N, Cropp, CB. Phylogeny of the genus *Flavivirus*. J Virol 1998; 72:73–83.

Kuno, G, Mitchell, CJ, Chang, GJ, Smith, GC. Detecting bunyaviruses of the Bunyamwera and California serogroups by a PCR technique. J Clin Microbiol 1996; 34:1184–1188.

Lanciotti, RS, Kerst, AJ, Nasci, RS, Godsey, MS, et al. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J Clin Microbiol 2000; 38: 4066–4071.

Larkin, MA, Blackshields, G, Brown, NP, Chenna, R, et al. Clustal W and Clustal X version 2.0. Bioinformatics 2007; 23: 2947–2948.

Lorono-Pino, M, Farfan-Ale, JA, Garcia-Rejon, JE, Lin, M, et al. Antibodies to influenza and West Nile viruses in domestic animals in the Yucatan Peninsula of Mexico. Vet Rec 2009 (In press).

Lorono-Pino, MA, Blitvich, BJ, Farfan-Ale, JA, Puerto, FI, et al. Serologic evidence of West Nile virus infection in horses, Yucatan State, Mexico. Emerg Infect Dis 2003; 9:857–859.

Lorono-Pino, MA, Farfan-Ale, JA, Zapata-Peraza, AL, Rosado-Paredes, EP, et al. Introduction of the American/Asian genotype of dengue 2 virus into the Yucatan State of Mexico. Am J Trop Med Hyg 2004; 71:485–492.

Morales-Betoulle, ME, Monzon Pineda, ML, Sosa, SM, Panella, N, et al. Culex flavivirus isolates from mosquitoes in Guatemala. J Med Entomol 2008; 45:1187–1190.

Pabbaraju, K, Ho, KC, Wong, S, Fox, JD, et al. Surveillance of mosquito-borne viruses in Alberta using reverse transcription polymerase chain reaction with generic primers. J Med Entomol 2009; 46:640–648.

Petersen, LR, Hayes, EB. West Nile virus in the Americas. Med Clin North Am 2008; 92:1307–1322, ix.

Ronquist, F, Huelsenbeck, JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 2003; 19:1572–1574.

Sang, RC, Gichogo, A, Gachoya, J, Dunster, MD, et al. Isolation of a new flavivirus related to cell fusing agent virus (CFAV) from field-collected flood-water *Aedes* mosquitoes sampled from a dambo in central Kenya. Arch Virol 2003; 148:1085–1093.

Schmaljohn, CS, Nichol, ST. Bunyaviridae. In: Knipe, DM, ed. *Fields Virology*, fifth edition. Philadelphia: Lippincott Williams and Wilkins, 2007:1741–1789.

Sexton, DJ, Rollin, PE, Breitschwerdt, EB, Corey, GR, et al. Life-threatening Cache Valley virus infection. N Engl J Med 1997; 336:547–549.

Sudia, W, Newhouse, V, Calisher, C, Chamberlain, R. California group arboviruses: isolations from mosquitoes in North America. Mosq News 1971; 31:576–600.

Weaver, SC. Host range, amplification and arboviral disease emergence. Arch Virol Suppl 2005; (19):33–44.

Wills, W, Pidcoe, V, Carroll, DF, Satz, JE. Isolation of California group arboviruses from Pennsylvania—1971,1972. Mosq News 1974; 34:376–381.

Address correspondence to:
Bradley J. Blitvich
2116 Veterinary Medicine
Iowa State University
Ames, IA 50011

E-mail: blitvich@iastate.edu