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Characterization of *Culex Flavivirus* (Flaviviridae) strains isolated from mosquitoes in the United States and Trinidad

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ABSTRACT

Recent reports indicate that flaviviruses similar to the *cell fusing agent virus* (CFAV) naturally infect a wide variety of mosquito species. These newly recognized insect-specific viruses comprise a distinct CFAV complex within the genus *Flavivirus*. Here, we describe the isolation and characterization of nine strains of *Culex flavivirus* (Cx FV), a member of the CFAV complex, from mosquitoes collected in the United States (East Texas) and Trinidad. Phylogenetic analyses of the envelope protein gene sequences of these nine mosquito isolates with those of other CFAV complex flaviviruses in GenBank indicate that the U.S. isolates group with CxFV isolates from Asia (Japan and Indonesia), while the Trinidad isolates are more similar to CxFV isolates from Central America. A discussion follows on the possible biological significance of the CFAV complex flaviviruses.
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Introduction

In 1975, Stollar and Thomas (1975) reported the isolation of a unique viral agent from the culture fluid of an *Aedes aegypti* cell line. This agent, when inoculated into an *Aedes albopictus* cell line, caused marked syncytia formation and was designated as a cell fusing agent virus (CFAV). The CFAV did not replicate in or cause cell fusion in mammalian cell cultures, so it was assumed to be an insect virus. Subsequent morphologic, biochemical and genetic studies (Igarashi et al., 1976; Cammissa-Parks et al., 1992; Kuno et al., 1998; Billoir et al., 2000) indicated that CFAV was a member of the genus *Flavivirus* (Flaviviridae) but that it was distantly related to other members of the genus. Thus, it was generally considered to be a curiosity.

Recent studies indicate that the CFAV and other genetically similar viruses are widely distributed in nature and occur in a variety of mosquito species. Cook et al. (2006) reported the isolation of over 40 isolates of CFAV group viruses from wild-caught adult *Aedes* and *Culex* species mosquitoes in Puerto Rico. Nucleic acid sequences homologous with the prototype CFAV were also found in homogenates of individual *Ae. aegypti* mosquitoes collected in Thailand (Kihara et al., 2007). Crabtree et al. (2003) reported the isolation of two strains of another unique CFAV group agent, designated Kamiti River virus (KRV), from larvae and pupae of *Ae. macintoshi* mosquitoes collected in Kenya. Hoshino et al. (2007) reported the isolation of yet another new CFAV group agent, named *Culex* flavivirus (CxFV), from *Cx. pipiens*, *Cx. tritaeniorhynchus* and *Cx. quinquefasciatus* mosquitoes collected in Japan and in Indonesia. Morales-Betoulle et al. (2008) reported the isolation of another CFAV group virus, designated CxFV Izabal 2006, from *Cx. quinquefasciatus* collected in Guatemala; and Farfan-Ale et al. (2009) found a high prevalence of infection with a similar virus in *Cx. quinquefasciatus* from southeastern Mexico.

Based on the aforementioned reports, we screened pools of mosquitoes collected in Harris County, Texas, U.S.A. and Champs Fleur, Trinidad, West Indies and isolated nine CxFV-like agents from *Cx. quinquefasciatus* and *Cx restuans*. The complete nucleotide sequence of one of the Texas isolates and envelope (E) nucleotide sequences of the other eight isolates were determined, and their phylogenetic relationships to other reported mosquito flaviviruses in the CFAV group were compared.

Results and discussion

Between February 16 and March 5, 2008, a total of 7223 female *Cx. quinquefasciatus* and 94 *Cx. restuans* were collected from Harris County, TX and processed for virus isolation in 339 and 49 pools,

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respectively. Upon examination of spot slides of the C6/36 cell cultures of these samples, it was observed that 19 of the *Cx. quinquefasciatus* pools (5.6%) and 18 of the *Cx. restuans* pools (36.7%) gave weak positive signals in IFAT with the SLEV polyclonal antibody, but not with the WNV antibody. Medium from these IFAT-positive cultures failed to react in the VecTest WNV/SLE antigen panel assay (Microgenetics). Further studies also showed a reaction in IFAT with JEV polyclonal antibody (Figs. 1A and B), but not with hyperimmune antisera prepared to DENV-2, yellow fever or Rio Bravo viruses. Attempts to propagate some of the suspected viruses in Vero cells or by intracerebral inoculation of newborn mice were unsuccessful. One of the IFAT-positive agents, TX 24518, produced marked syncytia in C6/36 cells after 6–7 days of incubation (Figs. 2A and B), similar to that described previously with the prototype CFAV (Stollar and Thomas, 1975). Transmission electron microscopy (TEM) done on C6/36 cells infected with TX 24518 showed giant multinucleated cells with virions 37–42 nm in diameter within the endoplastic reticulum of infected cells (Figs. 3A and B). This confirmed the presence of a virus in the culture, which had phenotypic (CPE) and morphologic characteristics like CFAV (Stollar and Thomas, 1975; Igarashi et al., 1976).

The pan-flavivirus primers MA and cFD2 were used in RT-PCR to determine the identity of the putative CFAV-like flaviviruses. All IFAT-positive cultures yielded PCR products of the expected sizes and, when sequenced, were shown to be homologous with sequences of CxFV isolates.

To better characterize the nine Houston and Trinidad mosquito isolates, TX 24518 was selected as the prototype and its full nucleotide sequence and deduced amino acids were determined. The genome of TX 24518 was found to be 10,837 nucleotides in length, with a 91 and 657 nucleotide UTR at the 5′ and 3′ ends, respectively. A single 10,089 nucleotide ORF (encoding 3363 amino acids) encodes three structural and seven nonstructural proteins, based on homology with other flaviviruses (Fig. 4). Subsequent Western blot analysis with the anti-JEV and anti-SLEV sera, using infected C6/36 cell lysate antigens or semi-purified virions of TX 24518, revealed reactions with a protein of approximately 47 kDa molecular weight, consistent with the putative CxFV envelope (E) protein (data not shown).

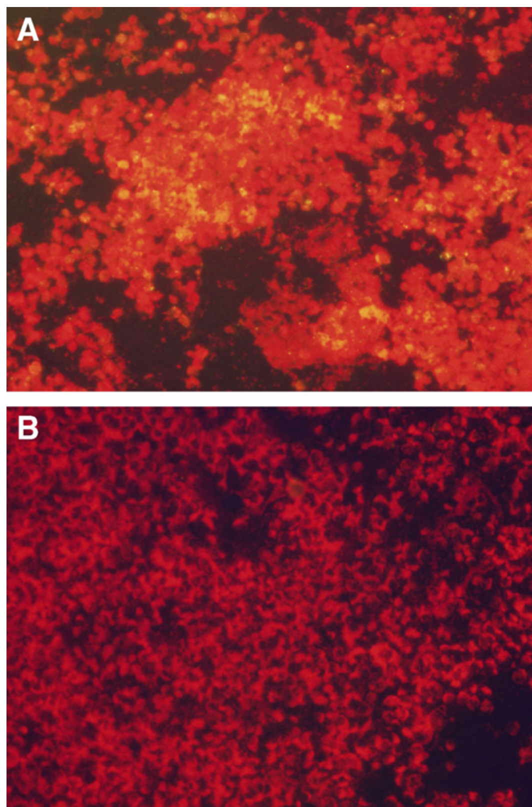

Fig. 1. (A) TX 24518 antigen in infected C6/36 cells, as detected by indirect fluorescent antibody technique (IFAT), using a Japanese encephalitis virus hyperimmune mouse ascitic fluid (HMAF). (B) C6/36 cell control (uninfected) stained by IFAT, using the same HMAF.

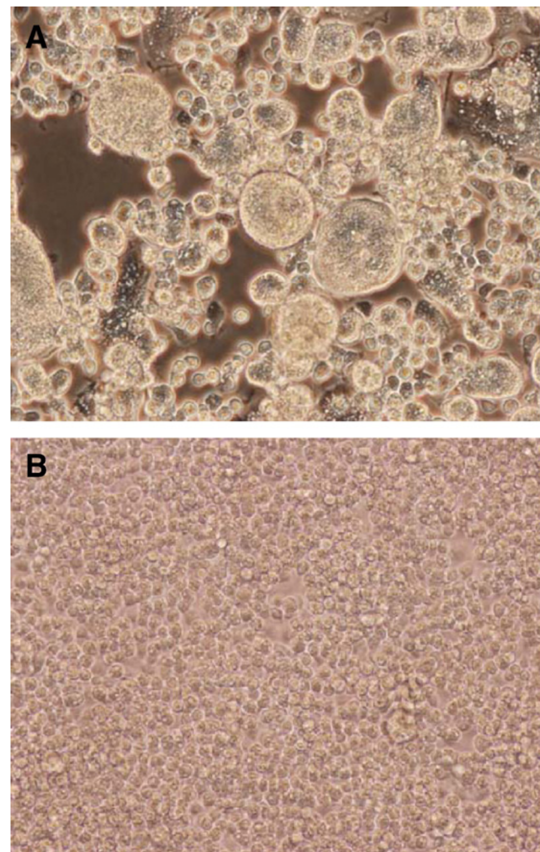

Fig. 2. (A) Phase contrast photomicrograph of C6/36 cells 7 days after infection with TX 24518. (B) Control (uninfected) C6/36 cells. Note the extensive cell fusion and syncytia formation in the infected cells (A).

The nucleotide and amino acid sequences of TX 24518 were compared with those of seven other mosquito-associated flaviviruses selected from the GenBank database. At the deduced polyprotein level, the TX 24518 genome shares 98.8% identity with the CxFV-Tokyo strain but only 46.8% and 40.8% with CFAV and KRV, respectively (Table 1). There was even less identity with three mosquito-borne flavivirus pathogens, WNV (29.1%), JEV (28.3%) and DENV-2 (28.4%) (Table 1). The envelope (E) protein of the other six Houston mosquito isolates (TX 24516, 24471, 24522, 24559, 24519 and 24284) also showed a high level of identity to the Japanese CxFV strains at the nucleotide (range 96.9 to 97.8%) and amino acid (range 98.1 to 99.5%) levels, but a lower homology to the Guatemalan Izabal strain (89.9 to 90.2% nucleotide and 96.5 to 97.2% amino acid) (data not shown). In contrast, the two Trinidad strains showed the highest identity with the Izabal strain (98.3–98.5% nucleotide and 99.5% amino acid) and a lower homology to Japanese strains (89.1–90.6% nucleotide and 96.5–97.4% amino acid). These data suggested that the Houston and Trinidad mosquito isolates represent new genetic variants of CxFV that are related to but distinct from CFAV and KRV. Furthermore, it appeared that the TX and Trinidad strains belong to different genetic subtypes of CxFV, being most closely related to the previously described Japanese or Central American strains of CxFV, respectively.

To better understand the genetic relationship and evolution of the Houston and Trinidad mosquito isolates, we performed Bayesian phylogenetic analysis of our nine isolates with 13 other flavivirus strains with complete envelope protein gene sequences (Fig. 5).

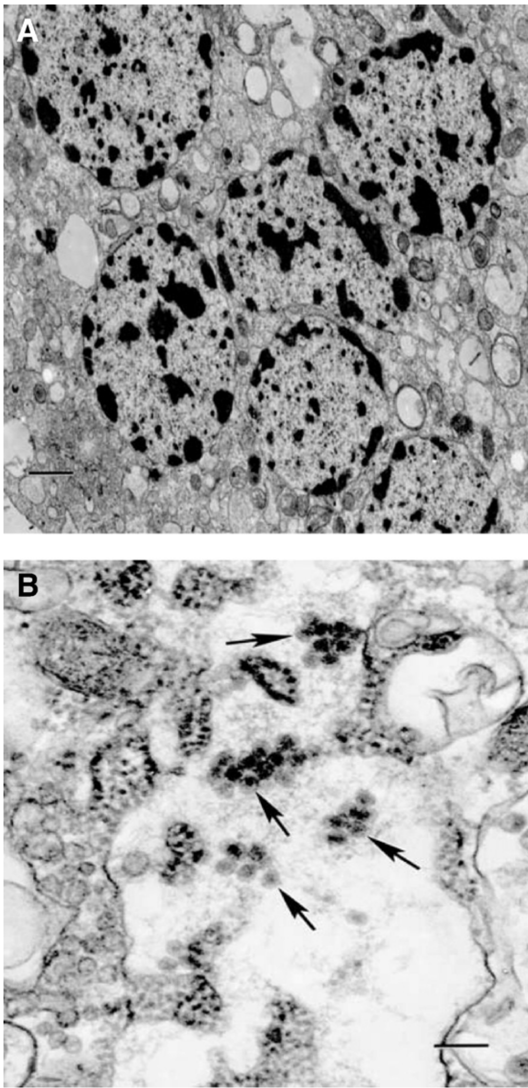

Fig. 3. (A) Transmission electron microscopy (TEM) photograph of a portion of a multinucleated giant cell (syncytium), demonstrating six nuclei, in a culture of C6/36 cells infected with TX 24518. Bar = 1 mm. (B) TEM photograph of a TX 24518-infected C6/36 cell, showing virions 37–42 nm in diameter (arrows) inside an enlarged cistern of granular endoplasmic reticulum.

Phylogenetic analyses using maximum parsimony and neighbor joining methods yielded trees with nearly identical topologies, differing only in some minor terminal branching orders (data not shown). Consistent with our initial analyses of nucleotide and amino acid sequence homologies, all of the Texas isolates formed a robust monophyletic group (85% clade probability), as did the two isolates from Trinidad (100% probability). However, the Texas isolates grouped with the Japanese-Indonesian *Culex* flaviviruses, while the two isolates from Trinidad were more closely related to the Guatemalan (Izabal) isolate. The criteria for species designations in the *Flavivirus* genus include a combination of genetic and antigenic characteristics as well as geographic, host, vector, ecologic and disease associations, many of which have not been characterized for the mosquito viruses considered here (Thiel et al., 2005). Kuno et al. (1998) proposed that >84% pairwise sequence identity can be used as the criterion for species of the members of the genus *Flavivirus*, since it correlated well with results obtained by neutralization testing. Neutralization tests were not attempted with the various members of the CFAV group. However, genetic comparisons suggest that 1) the *Culex* flavivirus group includes isolates from Guatemala, Trinidad, Texas, Japan and Indonesia; 2) cell fusing agent, and; 3) Kamiti River virus comprise 3 distinct flavivirus species. The levels of genetic differences among these three groups, 29% or higher in the envelope protein amino acid sequence identity, are comparable to those among many flavivirus species. The Trinidad/Guatemala and Asia/Texas clades we identified might comprise subtypes or genotypes within a putative *Culex* flavivirus species. The identification and analysis of additional strains of CxFV from other geographical regions would be required to determine whether these distinctions are genuine and to attempt to explain why North American strains of CxFV are more closely related to Asian strains than to Central American and Caribbean strains.

Table 2 shows the species composition, number of individuals and collection dates of the nine IFAT-positive mosquito pools included in this study. Three pools from Texas consisted of *Cx. restuans* females and six pools (from Texas and Trinidad) were comprised of *Cx. quinquefasciatus*. Two facts in Table 2 are noteworthy. First, two of the positive pools from Texas consisted of just single mosquitoes (TX 24219 and TX 24284), and two other positive pools (TX 24559 and TX 24471) consisted of only two and three individuals, respectively. These data suggest that the infection rate with CFAV-like agents in the Harris County *Culex* mosquito population was relatively high at this time, since these pools were collected at several different sites within the County over a 19-day period. We suspect that the sensitivity of our detection system (IFAT) was low and that some additional virus-positive pools were missed by this method. Other investigators (Cook et al., 2006; Kihara et al., 2007; Farfan-Ale et al., in press) have also reported high field infection rates with CSAV-group agents, as well as isolates from male mosquitoes, suggesting vertical transmission (Lutomiah et al., 2007).

The second interesting finding is the apparent seasonal activity of these viruses in Harris County. Houston has relatively mild winters, and there is adult *Culex* activity throughout the year. The abundance of *Culex* mosquitoes decreases in the cooler months (Nov.–March), but there are always gravid females present. During the cooler months, collections of *Cx. restuans* increase, whereas in the warmer months they decrease. We continued to sample *Culex* mosquitoes from April to August 2008, but did not detect any more flavivirus-positive mosquito pools. This finding might indicate that the activity of these viruses in the local *Culex* population is seasonal.

Despite their genetic similarity in the region under investigation, the seven Houston mosquito flaviviruses examined in this study showed phenotypic differences. Strain TX 24518 produced marked cytopathic effect and syncytia formation in C6/36 cells similar to that

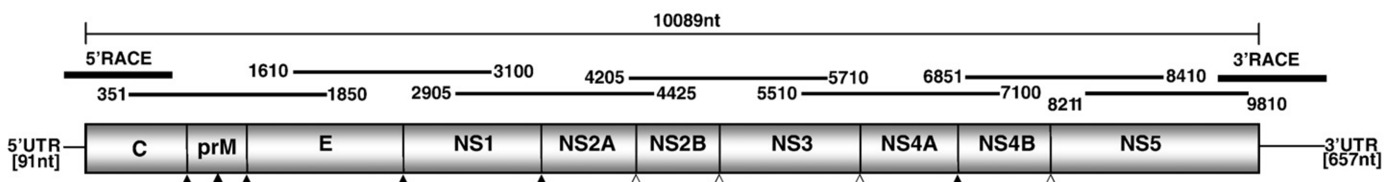

Fig. 4. Diagram of the genome organization and RT-PCR strategy used to sequence genome RNA of the Houston mosquito flaviviruses. Seven overlapping PCR amplicons representing the entire mosquito flavivirus genome, apart from the 5¢ and 3′ termini, were selected. RACE-PCR was performed for the 5′ and 3′ termini. The entire nucleotide length of polyprotein is shown with that of 5′ and 3′ untranslated regions (UTR). Both ▲ and Δ indicate the predicted signalase-like and NS2B-NS3 mediated cleavage sites, respectively. (Arrow indicates the trans-Golgi cleavage site).

Table 1
Amino acid and nucleotide sequence comparison (% sequence identity) of strain TX 24518 with selected mosquito-associated flaviviruses

TX 24518	C	prM	E	NS1	NS2A	NS2B	NS3	NS4A	NS4B	NS5	Polyprotein
	139aa	143aa	427aa	369aa	230aa	127aa	592aa	189aa	257aa	889aa	3362aa
CxFV-Tokyo	100[96.6]	98.6[95.1][a]	99.5[97.4]	100[97.4]	99.5[98.8]	98.4[98.1]	98.4[96.7]	98.9[97.3]	99.2[96.8]	98.6[96.8]	98.8[97.1]
	139aa	143aa	427aa	369aa	230aa	127aa	594aa	189aa	257aa	889aa	3362aa
CFAV	37.1[46.1]	69.2[62.7]	69.1[64.4]	39.3[43.2]	22.8[33.7]	17.5[31.4]	43.7[48.6]	22.0[33.0]	21.5[28.9]	59.4[66.2]	46.8[53.5]
	136aa	142aa	427aa	390aa	232aa	124aa	577aa	135aa	258aa	887aa	3341aa
KRV	28.6[35.1]	40.8[49.7]	33.6[41.3]	40.2[51.5]	22.2[31.3]	16.2[30.6]	45.1[52.2]	36.2[41.8]	25.3[33.2]	55.5[64.3]	40.8[48.6]
	143aa	143aa	432aa	390aa	232aa	124aa	577aa	168aa	261aa	887aa	3357aa
WNV	23.1[32.4]	19.6[25.3]	20.7[29.4]	25.9[33.2]	18.7[32.3]	15.5[31.2]	31.9[40.5]	19.0[26.4]	18.9[27.6]	44.3[52.5]	29.0[44.4]
	123aa	167aa	501aa	352aa	231aa	131aa	619aa	149aa	255aa	905aa	3433aa
SLEV	23.4[29.3]	18.5[25.6]	21.3[33.8]	26.2[29.2]	17.5[23.3]	15.8[27.6]	32.5[45.3]	20.2[34.3]	19.5[25.3]	44.8[51.4]	29.1[43.2]
	123aa	167aa	501aa	352aa	228aa	131aa	619aa	149aa	255aa	905aa	3430aa
JEV	25.5[28.4]	22.1[30.5]	20.6[29.3]	26.7[31.1]	21.4[26.3]	21.9[25.7]	32.3[41.9]	19.2[28.6]	18.6[25.4]	44.6[52.5]	28.3[44.1]
	127aa	167aa	500aa	352aa	227aa	131aa	619aa	126aa	255aa	905aa	3431aa
DEN2V	21.5[26.7]	18.4[23.2]	20.8[28.6]	22.8[25.3]	18.0[28.3]	23.9[32.3]	33.2[39.7]	22.5[27.5]	22.6[29.1]	44.1[53.4]	28.4[45.2]
	100aa	166aa	495aa	352aa	218aa	130aa	615aa	150aa	248aa	900aa	3391aa

[a] Percent nucleotide sequence homology shown in brackets.

described with the prototype CFAV, while the other six Houston mosquito isolates did not. Hoshino et al. (2007) reported that some but not all Japanese isolates of CxFV also produced CPE and cell aggregation. Similarly, two strains of KRV produced CPE in C6/36 cells (Sang et al., 2003), while CSAV did not (Cook et al., 2006). These observations indicate that CPE with syncytia formation in C6/36 cells is not a uniform characteristic of all viruses of the CFAV group.

The available data suggest that the CFAV group of flaviviruses is widely distributed and relatively common in *Aedes* and *Culex* mosquito populations of the world although there is currently no evidence that these agents infect or cause disease in vertebrates. Nonetheless, it is interesting to speculate on the significance of this newly recognized group of viruses. It has been suggested that the CFAV group of flaviviruses are specialized to adapt to mosquito hosts, and could thus represent primordial forms of the many arthropod-borne flaviviruses that cause disease in humans and animals (Hoshino et al., 2007). Crochu et al. (2004) reported the detection of CSAV sequences in the genome of laboratory-reared and wild *Ae. aegypti* and *Ae. albopictus* mosquitoes; and they hypothesized that these sequences had become integrated into the genomes of the mosquitoes following infection by a corresponding RNA virus (i.e. CFAV or a related CFAV group agent). If true, this finding has major implications regarding evolution, since it represents a novel method for horizontal gene transfer in eukaryotic cells.

Another intriguing question is, what effect does natural infection with a CFAV group agent have on the susceptibility of a mosquito to other flavivirus pathogens, such as dengue, yellow fever, West Nile or Japanese encephalitis viruses? Would a mosquito naturally infected with a CFAV group agent be refractory to superinfection with another flavivirus pathogen due to viral interference? Or would it be more susceptible? It is generally assumed that intrinsic differences in oral susceptibility and refractoriness to arbovirus infection in mosquitoes of a given species are largely determined by genetic factors (Black and Severson, 2005); but there may be others. Given the apparent

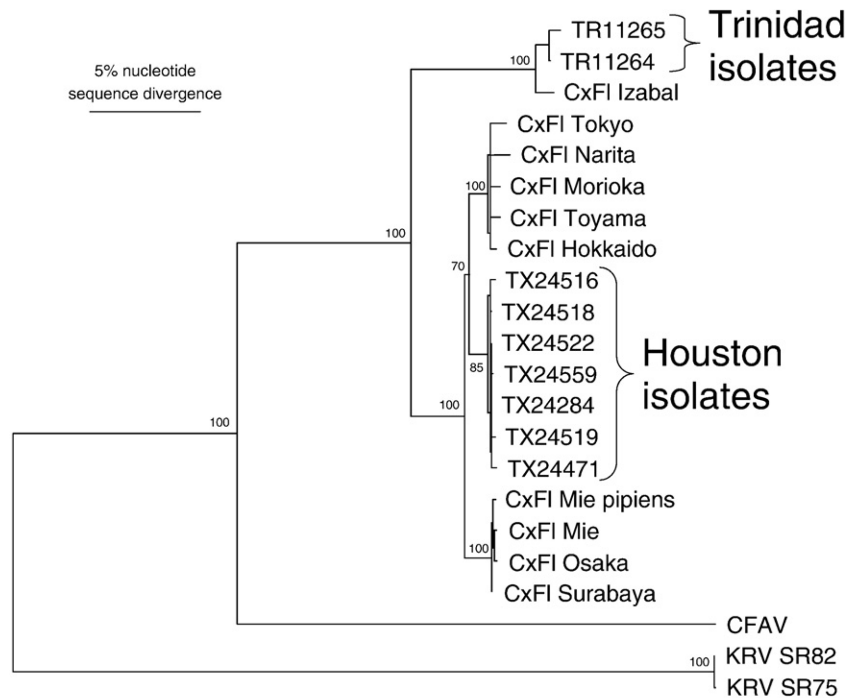

Fig. 5. MrBayes phylogenetic tree inferred for newly isolated mosquito flaviviruses from Houston, Texas (prefixed TX) and Trinidad (prefixed TR), CxFV from Japan (prefixed CFI), and homologous mosquito flavivirus sequences available in the GenBank library. The orientation of the tree was determined using midpoint rooting. Numbers at nodes indicate Bayesian probability values for clades to the right.

Table 2
Information on nine mosquito pools from Texas (TX) and Trinidad (TR) that yielded Culex flavivirus isolate

Virus strain (pool) number	*Culex* (*Cx.*) species	Number of mosquitoes in pool	Collection date
TX 24219	*Cx. restuans*	1	Feb. 16, 2008
TX 24284	*Cx. restuans*	1	Feb. 23, 2008
TX 24471	*Cx. restuans*	3	Mar. 1, 2008
TX 24516	*Cx. quinquefasciatus*	24	Mar. 5, 2008
TX 24518	*Cx. quinquefasciatus*	50	Mar. 5, 2008
TX 24522	*Cx. quinquefasciatus*	10	Mar. 5, 2008
TX 24559	*Cx. quinquefasciatus*	2	Mar. 5, 2008
TR 3115	*Cx. quinquefasciatus*	33	Feb. 25, 2008
TR 3116	*Cx. quinquefasciatus*	19	Feb. 25, 2008

frequency of CFAV group infection among mosquitoes in nature, this possibility should be investigated further.

Materials and methods

Cells

The C6/36 (*Ae. albopictus*) cell line was obtained from the American Type Culture Collection, Manassas, VA and was used for virus isolations and all subsequent virus propagations.

Mosquito collections

The Texas mosquitoes used in this study were collected as part of a long-term West Nile/St. Louis encephalitis virus surveillance program carried out jointly by the Harris County Mosquito Control Division and UTMB. The study area, which is located in the Houston metropolitan area, and methods have been previously described (Lillibridge et al. 2004). After collection, mosquitoes were sorted into pools, according to species, and processed for virus isolation. The mosquitoes yielding the viruses described in this report were collected during February and March of 2008.

The mosquitoes from Trinidad were collected on February 25, 2008, using a BioGents Sentinel trap (Maciel de Freites et al., 2006) baited with CO_2 and BG lure (Bioquip Products, Rancho Dominquez, CA) in the Champs Fleurs area. A total of 54 *Cx. quinquefasciatus* were collected and were divided into three pools which were frozen and transported on dry ice to UTMB where they were processed for virus isolation, as described below.

Virus isolation

Pools of female mosquitoes (*Cx. quinquefasciatus* or *Cx. restuans*) were homogenized in 1.0 ml of phosphate-buffered saline, pH 7.4, with 25% fetal bovine serum, using a TissueLyser (Qiagen, Valencia, CA) and 3 mm stainless steel beads. After centrifugation at 10,000 rpm in a microcentrifuge for 5 min, 100 µl of the supernatant was inoculated into single 12.5 cm^2 flasks with monolayer cultures of C6/36 cells. The cultures were maintained at 28 °C for 6–7 days and were examined every 2 days for evidence of viral cytopathic effect (CPE). If no CPE was observed, some of the cells were scraped from the surface of each flask and spotted onto 12-well glass slides for examination by indirect fluorescent antibody technique (IFAT).

IFAT

A standard IFAT was done, using specific mouse hyperimmune polyclonal antibodies prepared to WNV, SLEV and JEV. The procedures used for the IFAT (Tesh, 1979) and for preparation of hyperimmune ascitic fluids (Xu et al., 2007) were described before. The immunizing antigens for antibody production were prepared from brains of newborn mice inoculated intracerebrally with the NY385-99 strain of WNV, the Parton strain of SLEV and the Nakayama strain of JEV.

Transmission electron microscopy

Infected monolayers of C6/36 cells were fixed in a mixture of 2.5% formaldehyde and 0.1% glutaraldehyde in 0.05 M cacodylate buffer pH 7.2 containing 0.03% trinitrophenol and 0.03% $CaCl_2$ for at least 1 h at room temperature. Fixed cells could be kept in the fixative at 4 °C until further processing. After washing in 0.1 M cacodylate buffer, cells were scraped off the plastic, pelleted and processed further as a pellet. The pellets were post-fixed in 1% OsO4 in 0.1 M cacodylate buffer pH 7.2, *en bloc* stained with 2% aqueous uranyl acetate for 20 min at 60 °C, dehydrated in graded series of ethanol and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut on Leica-Reichart (Vienna, Austria), Ultracut S ultramicrotome, stained with lead citrate and examined in a Philips (Eindhoven, Netherlands) CM-100 electron microscope at 60 KV.

RT-PCR and nucleotide sequencing

Viral RNA was extracted from cell culture supernatants, using the QIAmp Viral RNA Mini kit (Qiagen). For initial amplification, the pan-flaviviral primer set (MA and cFD2; Kuno et al., 1998; Scaramozzino et al., 2001) for the region of NS5 was used. To amplify the complete nucleotide sequence of the viral genomic RNA, seven overlapping primer sets were designed to amplify nt 351 to 9810 (Fig. 4). Reverse transcription-polymerase chain reactions (RT-PCR) were performed using a reverse transcriptase, AMV (Roche, Nutley, NJ) and Extaq *pol* (Takara Bio, Otsu, Japan). The 5′ and 3′ end sequences were determined using GeneRacer kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The amplicons were purified using a QIAquick Gel Extraction kit (Qiagen) and submitted to the Protein Chemistry core laboratory at UTMB for sequencing, using BigDye version 3.1 cycle sequencing kits (Applied Biosystems) on an Applied Biosystems 3100 Genetic Analyzer. Sequence data for the entire genome of strain TX 24518 and the E gene regions of six other strains from TX and two strains from Trinidad were deposited in GenBank under accession numbers FJ502995 and FJ502996-FJ503003, respectively.

Genetic characterization of isolate TX 24518 and comparison with other flaviviruses

The nucleotide sequence and derived amino acid sequences of each viral protein of the TX 24518 isolate were compared with those of other selected flaviviruses, including CxFV (GenBank accession no. NC008604); CFAV (GenBank NC001564); KRV (GenBank NC005064); WNV (GenBank AF196835); SLEV (GenBank DQ525916); JEV (GenBank NC001437); and DENV-2 (GenBank NC001474). The CLC main workbench (Denmark) was used for multiple alignment and comparison.

Multiple alignment and phylogenetic analysis

CLAUSTALX (Thompson et al., 1997) was used to align the complete envelope protein gene sequences of the new mosquito flavivirus isolates with those of homologous mosquito flavivirus sequences available in the GenBank library. Phylogenetic trees were then inferred using maximum parsimony and neighbor joining programs implemented in the PAUP 4.0 software package (Swofford, 1998) and using MrBayes 3.1 for Bayesian analysis (Huelsenbeck and Ronquist, 2001). The neighbor joining analysis used, the HKY85 distance formula and the Bayesian analysis used a codon-based, general time reversible substitution model and 1,000,000 generations.

Acknowledgments

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References

Billoir, F., de Chesse, R., Tolou, H., de Micco, P., Gould, E.A., de Lamballerie, X., 2000. Phylogeny of the genus *Flavivirus* using complete coding sequences of arthropod-borne viruses and viruses with no known vector. J. Gen. Virol. 81, 781–790.

Black, W.C., Severson, D.W., 2005. Genetics of vector competence. In: Marquardt, W.C. (Ed.), Biology of Disease Vectors. Elsevier Academic Press, San Diego, CA, pp. 415–448.

Cammisa-Parks, H., Cisar, L.A., Kane, A., Stollar, V., 1992. The complete nucleotide sequence of cell fusing agent (CFA): homology between the nonstructural proteins encoded by CFA and the nonstructural proteins encoded by arthropod-borne flaviviruses. Virology 189, 511–524.

Cook, S., Bennett, S.N., Holmes, E.C., de Chesse, R., Moureau, G., de Lamballerie, X., 2006. Isolation of a new strain of the flavivirus cell fusing agent virus in a natural mosquito population from Puerto Rico. J. Gen. Virol. 87, 735–748.

Crabtree, M.B., Sang, R.C., Stollar, V., Dunster, L.M., Miller, B.M., 2003. Genetic and phenotypic characterization of the newly described insect flavivirus, Kamiti River virus. Arch. Virol. 148, 1095–1118.

Crochu, S., Cook, S., Attoui, H., Charrel, R.N., De Chesse, R., Belhouchet, M., Lemasson, J.J., de Micco, P., de Lamballerie, X., 2004. Sequences of flavivirus-related RNA viruses persist in DNA form integrated in the genome of *Aedes spp.* mosquitoes. J. Gen. Virol. 85, 1971–1980.

Farfan-Ale, J.A., Lorono-Pino, M.A., Garcia-Rejon, J.E., Hovav, E., Powers, A.M., Lin, M., Dorman, K.S., Platt, K.B., Bartholomay, L.C., Soto, V., Beaty, B.J., Lanciotti, R.S., Blitvich, B.J., 2009. 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. 80, 85–95.

Hoshino, K., Isawa, H., Tsuda, Y., Yano, K., Sasaki, T., Yuda, M., Takasaki, T., Kobayashi, M., Sawabe, K., 2007. Genetic characterization of a new insect flavivirus isolated from *Culex pipiens* mosquito in Japan. Virology 359, 405–414.

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17, 754–755.

Igarashi, A., Harrap, K.A., Casals, J., Stollar, V., 1976. Morphological, biochemical, and serological studies on a viral agent (CFA) which replicates in and causes fusion of *Aedes albopictus* (Singh) cells). Virology 74, 174–187.

Kihara, Y., Satho, T., Eshita, Y., Sakai, K., Kotaki, A., Takasaki, T., Rongsriyam, Y., Komalamisra, N., Srisawat, R., Lapcharoen, P., Sumroiphon, S., Iwanaga, S., Ushijima, H., Endoh, D., Miyata, T., Sakata, A., Kashige, N., Miake, F., Fukushi, S., Saijo, M., Kurane, I., Morikawa, S., Mizutani, T., 2007. Rapid determination of viral RNA sequences in mosquitoes collected in the field. J. Virol. Methods 146, 372–374.

Kuno, G., Chang, G.J.J., Tsuchiya, K.R., Karabatsos, N., Cropp, B., 1998. Phylogeny of the genus *flavivirus*. J. Virol. 72, 73–83.

Lillibridge, K.M., Parsons, R., Randle, Y., Travassos da Rosa, A.P.A., Guzman, H., Siirin, M., Wuithiranyagool, T., Hailey, C., Higgs, S., Bala, A.A., Pascua, R., Meyer, T., Vanlandingham, D.L., Tesh, R.B., 2004. The 2002 introduction of West Nile virus into Harris County, Texas, an area historically endemic for St. Louis encephalitis. Am. J. Trop. Med. Hyg. 70, 676–681.

Lutomiah, J.L.L., Mwandawiro, C., Magambo, J., Sang, R.C., 2007. Infection and vertical transmission of Kamiti river virus in laboratory bred *Aedes aegypti* mosquitoes. J. Insect. Sci. 55, 1–7.

Maciel de Freitas, R., Eiras, A.E., Lourenco de Oliveira, R., 2006. Field evaluation of the BG-Sentinel, a new trap for capturing adult *Aedes aegypti* (Diptera: Culicidae) Mem. Inst. Oswaldo Cruz 101, 321–325.

Morales-Betoulle, M.E., Monzon-Pineda, M.L., Sosa, S.M., Panella, N., Lopez, M.R., Cordon-Rosales, B.C., Komar, N., Johnson, B.W., 2008. Isolation of a Central American strain of *Culex* flavivirus in natural mosquito population from Guatemala. J. Med. Entomol. 45, 1187–1190.

Sang, R.C., Gichogo, A., Gachoya, J., Dunster, M.D., Ofula, V., Hunt, A.R., Crabtree, M.B., Miller, B.R., Dunster, L.M., 2003. 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. 148, 1085–1093.

Scaramozzino, N., Crance, J.M., Jouan, A., DeBriel, D.A., Stoll, F., Garin, D., 2001. Comparison of flavivirus universal primer pairs and development of a rapid, highly sensitive heminested reverse transcription-PCR assay for detection of flaviviruses targeted to a conserved region of the N55 gene sequences. J. Clin. Microbiol. 39, 1922–1927.

Stollar, V., Thomas, V.L., 1975. An agent in the *Aedes aegypti* cell line (Peleg) which causes fusion of *Aedes albopictus* cells. Virology 64, 367–377.

Swofford, D.L., 1998. PAUP*, Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, MA.

Tesh, R.B., 1979. A method for the isolation and identification of dengue viruses, using mosquito cell cultures. Am. J. Trop. Med. Hyg. 28, 1053–1059.

Thiel, H.J., Collett, M.S., Gould, E.A., Heinz, F.X., Houghton, M., Meyers, G., Purcell, R.H., Rice, C.M., 2005. Family Flaviviridae. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy. Classification and Nomenclature of Viruses. Eighth Report of the International Committee on the Taxonomy of Viruses. Elsevier Academic Press, San Diego, pp. 981–998.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAl_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

Xu, F., Liu, D., Nunes, M.R.T., Travassos da Rosa, A.P.A., Tesh, R.B., Xiao, S.Y., 2007. Antigenic and genetic relationships among Rift Valley fever virus and other selected members of the genus *Phlebovirus* (*Bunyaviridae*). Am. J. Trop. Med. Hyg. 76, 1194–1200.