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Short Communication

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Co-circulation of Flanders Virus and West Nile Virus in *Culex* Mosquitoes (Diptera: Culicidae) from Chicago, Illinois

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Abstract

West Nile virus (WNV) and Flanders virus (FLAV) co-occur in regions of North America. Because both viruses are maintained in a transmission cycle involving *Culex* mosquitoes and birds, screening mosquitoes for FLAV has been suggested as an enhancement to WNV surveillance and epidemic prediction. Using samples collected in 2010 and 2012 in Chicago, IL, USA, we demonstrate the presence of FLAV in four out of 287 (1.4%) *Culex* pools. We estimated minimum infection rates for WNV and FLAV to be 5.66 and 1.22 in 2010 and 8.74 and 0.61 in 2012, respectively. FLAV occurred 1 and 3 wk prior to the peak of WNV transmission in 2010 and 2012, respectively. FLAV sequences from Chicago were genetically diverse and phylogenetically representative of lineage A viruses from across the United States.

Key words: Flanders virus, West Nile virus, co-infection

Flanders virus (FLAV) is a single-stranded RNA hapavirus within the *Rhabdoviridae* family (Kokernot et al. 1969; Boyd 1972; Nasci et al. 2001). The prototype strain 61-7684 was first isolated in the town of Flanders in Long Island, NY in 1961 (Whitney 1964). FLAV is widely distributed throughout the United States and circulates in a bird-mosquito cycle in the eastern half of the United States (Whitney 1964; Mack et al. 1967; Sudia et al. 1967a; Sudia et al. 1967b; Chamberlain et al. 1969; Kokernot et al. 1969; Kokernot et al. 1974; Main et al. 1979; Rowley et al. 1983; Andre et al. 1985; Gilliland et al. 1995; Mitchell et al. 1996; Nasci et al. 2001; Wozniak et al. 2001; Takeda et al. 2003; Lucero et al. 2016), Utah (Crane et al. 1970), Canada (Hall et al. 1969; Thorsen et al. 1980; Belloncik et al. 1982), and Mexico (Sudia et al. 1975).

Similar to West Nile virus (WNV), avian hosts involved in transmission include house sparrows (*Passer domesticus* L., Passeriformes: Passeridae), red-winged blackbirds (*Agelaius phoeniceus* L., Passeriformes: Icteridae), and Northern cardinals (*Cardinalis cardinalis* L., Passeriformes: Cardinalidae) (Whitney 1964; Kokernot et al. 1969) and common mosquito vectors belong to the *Culex* genus (Diptera: Culicidae) (e.g., *Culex quinquefasciatus* Say, *Culex pipiens* L., *Culex restuans* Theobald, and *Culex tarsalis* Coquillett), all of which vector zoonotic encephalitic viruses (Sudia et al. 1967b; Kokernot et al. 1969; Nasci et al. 2001). In contrast to WNV, FLAV is not known to be pathogenic in humans (Kokernot et al. 1969; Lucero et al. 2016).

Because FLAV and WNV share the same hosts and vectors, there is renewed interest in patterns of FLAV and WNV co-occurrence in space and time. According to Lucero et al. (2016), FLAV circulates between April and October with a peak in June, approximately 10 wk prior to the peak of WNV in Tennessee. Spatially, WNV occurred within 3,000 m (1.86 miles) of FLAV-positive pools in Shelby County, Tennessee, with positive locations overlapping geographically during peak transmission (Lucero et al. 2016). FLAV may therefore serve as an 'early warning system' for WNV amplification (Lucero et al. 2016).

We collected and tested *Culex* mosquito pools collected in 2010 and 2012 from suburban Chicago, IL for FLAV. Our goals were to assess whether FLAV might co-occur with WNV and whether temporal patterns reported by Lucero et al. (2016) in the greater Memphis area of Tennessee might also occur in the greater Chicago area of Illinois. We also conducted a phylogenetic analysis to determine the relationship of FLAV detected in Chicago to other lineages circulating in the United States.

Methods

Mosquito Collection

Mosquitoes were collected from the Chicago, IL region in 2010 and 2012, 2 yr with high WNV infection rates in mosquitoes. Briefly, mosquitoes were collected using CO_2-baited light traps and infusion-baited gravid traps for one night per week between June and October for both years. Mosquitoes were then identified and pooled based on their species, date, unique trap location, and blood-fed status as described in Hamer et al. (2008). Given the inherent uncertainty in distinguishing *Cx. pipiens* and *Cx. restuans* morphologically (Harrington and Poulson 2008), separation of these two species was not attempted during the creation of *Culex* spp. pools.

We previously reported WNV minimum infection rate (MIR) values of 5.66 and 8.74 per 1,000 mosquitoes, respectively, in 2010 and 2012 (Shand et al. 2016). Because Lucero et al. (2016) found FLAV prior to the amplification of WNV, we selected for FLAV testing a subset of mosquito pools prior to and during the peak of WNV amplification. We tested 130 and 82 *Culex* pools prior to and 17 and 58 pools during the peak WNV season in 2010 and 2012, respectively.

RNA Isolation and Virus Testing

Culex mosquito pools were homogenized in 800 µl of lysis binding solution concentrate (Thermo Fisher Scientific, Waltham, MA) with three #7 steel shots on a vortex mixer (VWR International, Radnor, PA) followed by centrifugation at 14,000 rpm for 2 min at room temperature. Resulting supernatant (115 µl) was used as starting material for RNA isolation using the MagMax Viral RNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA). Extracted RNA was stored at –80°C pending further testing. *Culex* pools were tested for WNV infection using a published quantitative reverse-transcriptase TaqMan assay (Lanciotti et al. 2000). Mosquitoes were tested for the presence of FLAV using a conventional reverse transcriptase PCR (RT-PCR) assay targeting a 486-bp region of the U1 gene followed by gel electrophoresis. For each reaction, 2.5 µl of extracted RNA was added to 22.5 µl of master mix containing Promega GoTaq Flexi DNA Polymerase and a concentration of 50 µM for each primer (FLAV-U1-F (forward, 5′ → 3′): TAG CAC TTG TAT CAG CCC AT; FLAV-U1-R (reverse, 5′ → 3′): GTT CAC TAA CTG TTC CCT TTT G) to create a 25-µl total volume reaction. The thermal cycling conditions for the reaction were 42°C for 45 min, 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 48°C for 30 s, 72°C for 1 min, and a final extension step of 72°C for 7 min and held at 4°C. FLAV positive control was obtained from the World Reference Center for Emerging Viruses and Arboviruses (University of Texas Medical Branch). The positive control was originally detected in a *Cx. quinquefasciatus* mosquito pool collected from Harris County, TX in 2005 (accession no. KF028716; Allison et al. 2014); to assess congruence, we sequenced this same isolate.

Amplicons were visualized on a 2% agarose gel and then purified using ExoSAP-IT PCR Product Cleanup (Affymetrix, Santa Clara, CA). Purified samples were sent to Eton Bioscience (San Diego, CA) for Sanger sequencing. To verify the presence of FLAV, newly obtained sequences were characterized using NCBI BLAST. MIR values for each virus were calculated using the maximum likelihood estimation (MLE) method from the CDC Excel Add-In (Biggerstaff 2009).

Phylogenetic Analysis of FLAV

To examine the phylogenetic position of FLAV circulating in Chicago, IL, we aligned new FLAV sequences using the Clustal-W method in Geneious version 9.1.8 (Thompson et al. 1994; Kearse et al. 2012). Alignments were analyzed using RAxML (Rapid Axelerated Maximum Likelihood) Blackbox server (Stamatakis 2014) to obtain a maximum likelihood tree with statistical confidence of groupings based on 10,000 bootstrap replicates of the data (Felsenstein 1985), with Hart Park Virus (HPV) strain AR7C as the outgroup (accession no. KM205011.1). In addition, 61 sequences of the U1 gene of FLAV lineages A and B reported in Allison et al. (2014) (accession nos. KF028661–KF028763) were included. All four unique sequences produced during this project and used in the phylogenetic analysis were deposited in GenBank (accession nos. MG844997–MG845000) (Table 1).

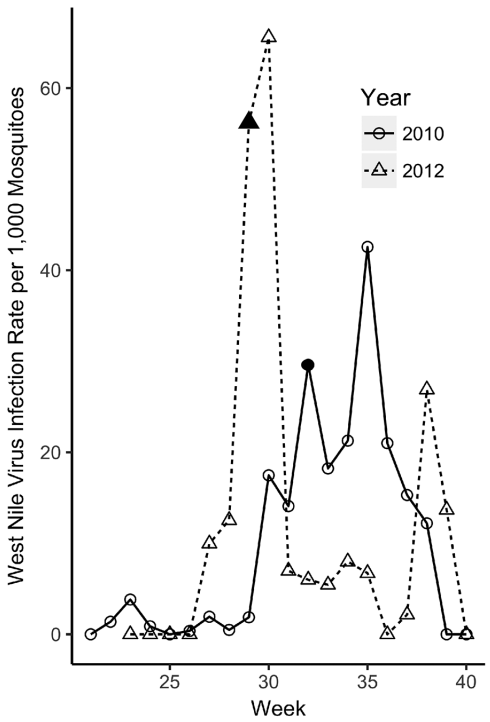

Fig. 1. *Culex* infection rate with West Nile Virus for 2010 and 2012 in Chicago, IL. The filled shapes denote the weeks when the FLAV-positive mosquito pools were collected.

Table 1. Flanders Virus positive pools of *Culex* spp. mosquitoes

Accession no.	Mosquitoes	Trapping date (wk)	WNV result	FLAV lineage
MG844997	25	8/12/2010 (32)	Positive	A
MG844998	50	8/12/2010 (32)	Positive	A
MG844999	40	8/12/2010 (32)	Positive	A
MG845000	22	7/18/2012 (29)	Positive	A

Cx. pipiens and *Cx. restuans* were not separated.

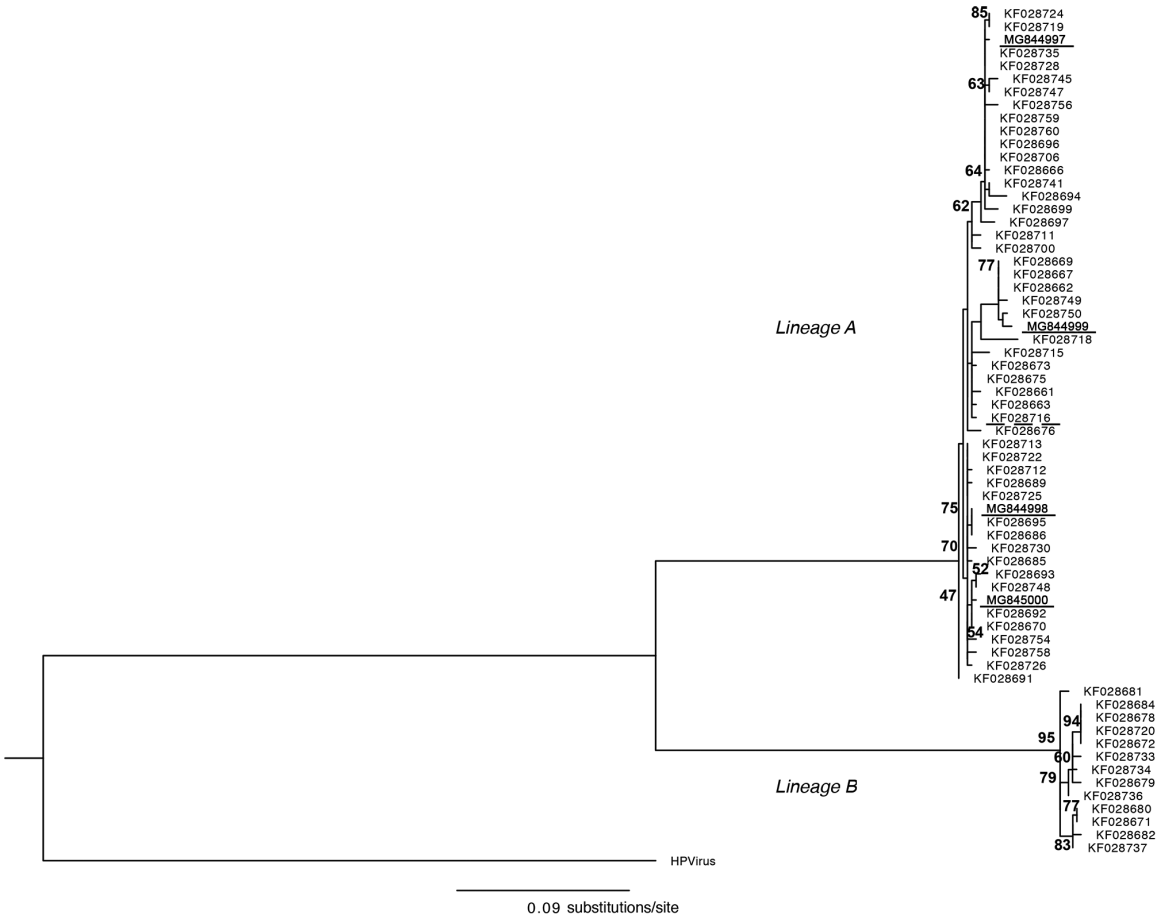

Fig. 2. Maximum likelihood (ML) phylogeny based on a 486-bp region of the FLAV U1 gene. The phylogenetic tree was rooted with Hart Park Virus (HPVirus) strain AR7C (accession no. KM205011.1) and includes four FLAV sequences from Chicago generated in this study represented with solid underlines (accession nos. MG844997–MG85000) as well as 61 publically available sequences of the Flanders virus U1 gene from the United States (accession nos. KF028661–KF028763). The positive control is highlighted with a dashed underline (accession no. KF028716). Bootstrap values are based on 10,000 ML replicates generated in RAxML (Stamatakis 2014) and values above 50.0 are shown.

Results and Discussion

FLAV and WNV Infection Rates and Co-occurrence

In total, we tested 287 *Culex* spp. pools for FLAV, representing 4,118 individual mosquitoes. These consisted of 69 WNV-positive pools and 78 WNV-negative pools from 2010 and 38 WNV-positive pools and 102 WNV-negative pools from 2012. Four total mosquito pools were positive for FLAV by PCR of the U1 gene and all were collected using gravid traps (Table 1). Our FLAV-positive pools were also previously positive for WNV. Three of these pools were collected on 8 August 2010. One was collected from Holy Sepulchre Cemetery, Alsip, IL and two were collected from an American robin (*Turdus migratorius*) communal roost in a natural area adjacent to interstate 294 in Alsip, IL. The remaining positive pool was collected on 18 July 2012 in a residential area of Alsip, IL. All four samples were collected within a 2-km^2 area of the study site (Hamer et al. 2014). The MIR values for FLAV in 2010 and 2012 were 1.22 and 0.61 per 1,000 *Culex* sp. mosquitoes, respectively. Because we did not separate *Cx. restuans* and *Cx. pipiens*, we were unable to determine which of these two species were infected with FLAV or WNV. However, concurrent blood meal analyses in the same region from 2005 to 2012 (Medeiros et al. 2015) determined that *Cx. pipiens* represents 95% of the *Culex* mosquito community (with *Cx. restuans* representing the remaining 5%) during the July to August time period (Hamer et al., unpublished data).

The appearance of FLAV prior to WNV was consistently reported in Tennessee by Lucero et al. (2016). FLAV and WNV would appear in spring and late spring and then peak in summer and late summer, respectively. The same study also found that peak FLAV occurrence preceded peak WNV occurrence by 10.2 wk in Shelby County, TN. Based on these results, the authors hypothesized that FLAV might be useful for WNV surveillance, as an 'early warning system'. Our results are broadly consistent with this idea but show less of a lag between FLAV and WNV appearance, with FLAV occurring only 1 and 3 wk prior to the peak of WNV in 2010 and 2012, respectively (Fig. 1). Furthermore, we found that only 1.4% of our samples were positive for FLAV, suggesting minimal amplification of this virus in the Chicago metropolitan area. This observation may have been influenced by the molecular technique used in this study (conventional RT-PCR), which some studies have found to be less sensitive than quantitative RT-PCR (Lanciotti et al. 2000) while others have found them to be similar (Bastien et al. 2008). Additionally, screening of additional pools for FLAV would have improved the spatiotemporal resolution of our study. Our current data suggest that the use of FLAV as a WNV 'early warning system' would not be efficient in this region.

Nevertheless, co-circulating pathogens have been identified as possible modulators of WNV transmission. For example, the insect-specific *Culex* flavivirus (CxFV) co-occurs with WNV (Crockett et al. 2012; Newman et al. 2017) and alters WNV transmission

Table 2. Flanders Virus positive pools from Chicago, IL and their most phylogenetically similar variants based on a 486 position alignment of the U1 gene

Accession no.	Strain origin	% Similarity	Accession number
MG844997	Chatham County, GA	99.8%	KF028696, KF028706, KF028735, KF028759, KF028760
	Harris County, TX	99.8%	KF028728
MG844998	Chatham County, GA	100.0%	KF028686
	Fulton County, GA	100.0%	KF028695
MG844999	Clayton County, GA	99.4%	KF028662
	DeKalb County, GA	99.4%	KF028750
	Lowndes County, GA	99.4%	KF028669
	Jefferson County, WV	99.4%	KF028667
MG845000	Chatham County, GA	99.8%	KF028670
	Fulton County, GA	99.8%	KF028692

(Kent et al. 2010) and flight behavior (Newman et al. 2016) in *Culex* mosquitoes. Interactions between WNV and FLAV therefore merit further investigation.

Phylogenetic Analysis of FLAV

There are currently two sympatric co-circulating lineages of FLAV (Lineage A and Lineage B) found throughout the United States (Allison et al. 2014). We found that the four FLAV-positive sequences identified in this study were nested within Lineage A and shared >97% similarity to samples within this lineage and with each other (Fig. 2, Table 2). Our samples were approximately 85% similar to Lineage B, which correlates with the approximate nucleotide divergence amount between Lineages A and B of the U1 gene reported in Allison et al. (2014). The four isolates from the Chicago study area span nearly the full phylogenetic diversity of Lineage A, even though our study area is small (Hamer et al. 2014). This pattern is similar to what we have previously documented for WNV, which displays approximately as much genetic diversity within this small geographic area in the suburbs of Chicago, Illinois, as across all of North America (Bertolotti et al. 2008; Amore et al. 2010).

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