Mercadeo Virus: A Novel Mosquito-Specific Flavivirus from Panama

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Abstract. Viruses in the genus *Flavivirus* (family *Flaviviridae*) include many arthropod-borne viruses of public health and veterinary importance. However, during the past two decades an explosion of novel insect-specific flaviviruses (ISFs), some closely related to vertebrate pathogens, have been discovered. Although many flavivirus pathogens of vertebrates have been isolated from naturally infected mosquitoes in Panama, ISFs have not previously been reported from the country. This report describes the isolation and characterization of a novel ISF, tentatively named Mercadeo virus (MECDV), obtained from *Culex* spp. mosquitoes collected in Panama. Two MECDV isolates were sequenced and cluster phylogenetically with cell-fusing agent virus (CFAV) and Nakiwogo virus (NAKV) to form a distinct lineage within the insect-specific group of flaviviruses.

INTRODUCTION

Viruses in the genus *Flavivirus* (family *Flaviviridae*) include many arthropod-borne viruses (arboviruses) of public health and veterinary importance.¹ On the basis of their vector and host associations, the flaviviruses can be divided into four broad groups: tick-borne, mosquito-borne, insect-specific, and no-known (arthropod) vector.^{2,3} Flaviviruses in the tick- and mosquito-borne groups generally circulate in alternating arthropod–vertebrate cycles; viruses in the insect-specific group infect arthropods but do not appear to infect vertebrates; and viruses included in the no known vector (NKV) group infect vertebrates but have no known arthropod association.

The first insect-specific flavivirus (ISF) to be reported was the cell-fusing agent virus (CFAV).^{4,5} The virus was so named because it produced cell fusion and syncytia formation in infected cultures of mosquito cells. Since 1975, many other ISFs have been detected in a wide variety of mosquito species and from many different geographic regions of the world.^{6–9} The relatively recent recognition of the multitude of ISFs circulating in nature has been due largely to the availability of mosquito cell cultures (principally the C6/36 *Aedes albopictus* cell line) and the development of RNA amplification and sequencing techniques. In general, the ISFs are not detectable by classical methods of arbovirus isolation and identification (i.e., inoculation of vertebrate cell cultures or laboratory animals and serology). The realization that ISFs are so abundant in mosquitoes worldwide and that some are closely related antigenically and genetically with important flavivirus pathogens raises interesting new questions as to their evolutionary origin, their potential to emerge as novel vertebrate pathogens, and their effect on the vector competence of their insect hosts for certain other flavivirus pathogens.

The purpose of the present communication is to report the isolation and genetic characterization of a novel ISF, designated Mercadeo virus (MECDV), obtained from *Culex* spp.

mosquitoes collected in Panama in 2011 during retrospective field studies in a region where an arbovirus encephalitis outbreak had occurred the year before.¹⁰

MATERIALS AND METHODS

Study sites. Mosquito collections were conducted in 2011 in Darien Province (7°38'0" N to 76°57'0" W), in eastern Panama, close to the border with Colombia, in the same region where a human and equine outbreak of alphaviral encephalitis had occurred during 2010.¹⁰ The collection localities were El Real de Santa Maria, Santa Librada, and Los Pavitos, in the municipality of Chepigana. There are two major wetland habitats at these sites. The first, Bayano Lake, covers an area of 350 km² and is located in the region of Choco/Darien. The vegetation of the region is classified as moist tropical forest with 85% relative humidity, 2,300 mm annual rainfall, and a mean temperature of 26°C. The second collecting site, Matusagarati Lagoon, has an area of 140 km², is located near the communities of Aguas Calientes and Yaviza in the Chepigana area of Darien Province, and has a similar vegetation classification (moist tropical forest), with a mean average temperature of 25°C and annual rainfall of 2,000 mm. The rainy season at both sites lasts for period of 8–9 months (May–December) and the dry period usually occurs for 3–4 months (January–April).¹¹

Collection methods. Mosquito collections were made in February, May, and September of 2011 for three consecutive days during each month of collection. At each collection site, two type of traps were placed from 6:00 PM to 6:00 AM at the edge of the forest, distributed equally in peridomestic and wooded areas near houses at a height of 1.5 m above ground level.¹² In each village, 10 households were also selected, where 10 Centers for Disease Control and Prevention light traps baited with octanol as an attractant and four encephalitis vector survey (CO₂) mosquito traps (BioQuip Products, Rancho Dominguez, CA) were placed. The following day, mosquitoes were collected in the field, transported to the base camp where they were anesthetized, identified to the genus level using taxonomic keys¹³ and then were placed in cryovials for storage in liquid nitrogen.

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Virus isolation. Mosquitoes were grouped into pools of 20 individuals by genus and collection site. Mosquito pools were homogenized in 2 mL of minimum essential medium supplemented with 1% penicillin and streptomycin and 20% fetal bovine serum, using a TissueLyser (Qiagen, Hidden, Germany). After centrifugation, 200 μ L of the supernatant was inoculated in two 12.5-cm² plastic tissue culture flasks of Vero and C6/36 cells. After absorption for 2 hours at 28°C (C6/36) or 37°C (Vero), 5 mL medium was added to each flask, and the cells were incubated at the respective temperatures and observed daily for evidence of viral cytopathic effect (CPE).

Transmission electron microscopy. C6/36 cells infected with mosquito isolates ER-M10 and ER-M31 were processed as described previously.¹⁴ Ultrathin sections were cut on a Leica EM UC7 ultramicrotome (Leica Microsystems, Buffalo Grove, IL), stained with lead citrate, and examined in a Philips 201 transmission electron microscope at 60 kV.

Preparation for viral RNA extraction. C6/36 cells grown to 90% confluence in T25 culture flasks were infected with isolate ER-M10 or ER-M31. The virus harvest and isolation of viral RNA (vRNA) for next-generation genome sequencing was processed as described previously.¹⁴

Construction of RNA library. vRNA (0.1–0.2 μ g) was fragmented by incubation at 94°C for 8 minutes in 19.5 mL fragmentation buffer (15016648; Illumina, San Diego, CA). First and second strand synthesis, adapter ligation, and amplification of the library were performed using the TruSeq RNA Sample Preparation Kit under conditions prescribed by the manufacturer (Illumina). Samples were tracked using the “index tags” incorporated into the adapters as defined by the manufacturer.

Sequence assembly and analysis. A sequencing library was prepared from the sample RNA using an Illumina TruSeq RNA v2 Kit, following the manufacturer’s protocol. The sample was sequenced on a HiSeq 1000 using the 2 $\times$ 50 paired-end protocol. Reads in FASTQ format were quality filtered, and any adapter sequences were removed using Trimmomatic software.¹⁵ The de novo assembly program ABySS¹⁶ (Michael Smith Genome Sciences Centre, Vancouver, Canada) was used to assemble the reads into contigs, using several different sets of reads, and k values from 20 to 40. A nearly full-length contig was obtained from 250,000 reads and a k value of 25. Reads were mapped back to the contig using Bowtie 2 (John Hopkins University, Baltimore, MD),¹⁷ and visualized with the Integrated Genomics Viewer¹⁸ (Broad Institute, Boston, MA) to verify that the assembled contig was correct. The reads obtained by sequencing on a HiSeq 1000 were mapped back to the contig (obtained from 250,000 reads) to verify that the assembled contig was correct. For ER-M31, 6% of the reads were mapped to the viral contig, resulting in about 120,000 reads mapped out of about 2 million total, whereas for ER-M10 about 2.8% of the reads in the sample were mapped to the viral contig, resulting in about 250,000 reads mapped out of about 9 million total. Pre-filtering of reads to remove host sequence enhanced the assembly process. Assembly was carried out using a FASTA file of *Ae. albopictus* sequences to remove host DNA from the assembly thus reducing the number of contigs present.

Nucleotide sequence accession numbers. The complete genome sequences of two MECDV strains were determined in this study and were assigned GenBank accession numbers

KP688057 and KP688058 for ER-M31 and ER-M10, respectively. The GenBank accession numbers for the genome sequences of select flaviviruses used in the phylogenetic analyses are listed in Supplemental Table 1.

Phylogenetic analysis. Selected flavivirus sequences representing a 2,757 nucleotide (nt) segments of the polymerase gene were downloaded from GenBank. These sequences, together with the corresponding sequence of MECDV, were combined and aligned manually using the Se-Al application based on amino acid sequence alignments. A neighbor-joining tree was built based on this alignment using PAUP* v4.0b package (Sinauer Associates, Sunderland, MA) as a guide tree. Overly sampled sequences from a single location and year were deleted to accelerate the analysis without sacrificing overall genetic diversity. This led to a final dataset of 77 samples. A maximum likelihood (ML) tree was then inferred using PAUP* based on the best-fit substitution model estimated from Modeltest version 3.06.¹⁹ The optimal ML tree was estimated using the appropriate model and a heuristic search with tree-bisection-reconstruction branch swapping and 1,000 replicates, estimating variable parameters from the data, where necessary. Bootstrap replicates were calculated for each dataset under the same models mentioned above. In addition, Bayesian analysis was undertaken using MrBayes v3.1,^{20,21} and datasets were run for 10 million generations until they reached congruence. The models used were HKY+G and HKY+I+G. Trees obtained by either analysis exhibited similar topologies.

RESULTS

Culture and growth characteristics. Two homogenates of *Culex* sp. mosquitoes (ER-M10 and ER-M31) produced visible CPE (rounding, clumping, and detachment of cells) 6 days after inoculation into C6/36 cultures. In contrast, no CPE was observed after 14 days of incubation in Vero cell cultures and no vRNA replication was detected by reverse transcriptase polymerase chain reaction (RT-PCR) (data not shown). Some of the culture fluid from the CPE-positive mosquito cells was subsequently cultured again in Vero and baby hamster kidney cells, but no CPE was observed. However we were able to make a second passage in C6/36 cells. Approximately 20 μ L of the infected C6/36 culture fluid from ER-M10 and ER-M31 were also each inoculated intracranially into a litter ($N = 10$) of newborn ICR mice, but no illness or death occurred after 14 days.

Virus ultrastructure. In ultrathin sections of C6/36 cells infected with ER-M10 and ER-M31, an unusual cytopathology was observed with significantly expanded cisterns of granular endoplasmic reticulum containing long microtubules, sometimes up to several micrometers long (Figure 1A and D). In cross-sections these tubules were ~30 nm in diameter (Figure 1E). The same cisterns could also contain virus particles ~45 nm in diameter and smooth membrane structures (SMSs) (Figure 1D). Virions ~45 nm in diameter were regularly found inside enlarged cisterns of granular endoplasmic reticulum (Figure 1B and C), as well as SMSs typical for flaviviruses (Figure 1B, D, and E).

Phylogenetic analysis. Several consensus trees were obtained based on ML and Bayesian analyses, which exhibited similar topologies. Here we only show the trees generated by the ML method (Figure 2). In the ML tree of 77 flaviviruses, based on a 2,757 nt segment of the polymerase gene sequences, isolates

FIGURE 1. Ultrastructure of ER-M31 and ER-M10 flaviviruses in ultrathin sections. (A) Portion of ER-M31-infected C6/36 cell demonstrating long microtubules within expanded cisterns of granular endoplasmic reticulum (arrow) (N = fragment of host cell nucleus, bar = 1 μ m). (B) Virions (v) and smooth membrane structures (SMS, thin arrows) within enlarged cisterns of granular endoplasmic reticulum in a cell infected with ER-M31 virus (bar = 100 nm). (C) Virions of ER-M10 virus within enlarged cisterns of granular endoplasmic reticulum (bar = 100 nm). (D) Enlarged fragment of Figure 1A showing long microtubules, virions (v) and SMS (thin arrows) inside enlarged cisterns of granular endoplasmic reticulum (N = portion of host cell nucleus, bar = 0.5 μ m). (E) Portion of C6/36 cell infected with ER-M31 virus demonstrating microtubules within enlarged cisterns of granular endoplasmic reticulum (arrow), their cross-sections (open arrow), virions (v), and SMS (thin arrow) (N = fragment of host cell nucleus, bar = 100 nm).

ER-M10 and ER-M31 cluster together as a distinct lineage within the insect-specific group of flaviviruses, which includes CFAV, *Culex flavivirus* (CxFV), Quang Binh virus (QBV), and NAKV (Figure 2). Both MECDV isolates are closely

related in this phylogeny with CFAV and NAKV, while the high bootstrap support (> 90%) indicates that these three viruses are still distantly related. Although fine-scale resolution of the evolutionary history of ER-M10 and ER-M31 is not possible based on these data, their dissimilarity strongly suggests that they represent a novel virus species.

Genome organization. The size of the positive sense, single-strand genomes of the two identified viruses were 10,938 nt long. A single open reading frame of 10,212 nt is flanked by untranslated regions (UTRs) at the 5' and 3' ends. The 5'-UTR is 88-nt long, while the 726-nt 3'-UTR lacks the classical polyadenylation site.²²

DISCUSSION

As an increasing number of novel ISFs have been discovered and genetically characterized, it has become apparent that the ISFs form two distinct clades in the phylogeny of the genus *Flavivirus* (Figure 2). One clade, which appears as a distant branch off of the main flavivirus phylogenetic tree, includes CFAV,²³ NAKV,²⁴ QBV,²⁵ CxFV,²⁶ Calbertado virus (CLBOV),²⁷ Kamiti River virus (KRV),²⁸ *Aedes flavivirus* (AeFV),²⁹ Palm Creek virus (PCV),⁸ and MECDV. A second group of ISFs is placed within the main clade of mosquito-borne flaviviruses (shown in Figure 2 within the bracket labeled "vertebrate host unknown") that includes Nanay virus (NANV),³⁰ Nouname virus (NOUV),³¹ Donggang virus (DGV),³² Lammi virus (LAMV),³³ Barkedji virus (BJV),³⁴ Nhumirim virus (NHUV),³⁵ Chaoyang virus (CHAOV),³⁶ and Marisma mosquito virus (MMV).³⁷

The continued discovery of novel ISFs in a wide range of mosquito species from diverse geographic regions indicates that these viruses are relatively common in mosquito populations worldwide. The available data suggest that ISFs are maintained in their mosquito hosts by vertical transmission and without causing deleterious effects.^{9,38,39} In this regard, they behave like symbionts and constitute part of the insect's microbiome. Therefore, it seems likely that these symbiotic viruses may affect vector competence for arboviruses.

Studies with several *Culex* species have suggested that mosquitoes infected with CxFV were less susceptible to West Nile virus infection than were control (CxFV-free) mosquitoes.^{40,41} Similarly, in vitro experiments with Palm Creek virus indicated that it reduced replication of Kunjin and Murray Valley viruses in dually infected *Ae. albopictus* (C6/36) cells.⁸ A recent article by Kenney and others³⁵ reported that Nhumirim virus also significantly reduced replication of West Nile, Japanese encephalitis, and St. Louis encephalitis viruses in vitro. In each of these reports, infection with a particular mosquito-specific flavivirus reduced replication of a mosquito-borne flavivirus pathogen. The mechanism of this reduction is unknown but competitive inhibition as well as induction of the RNA interference (RNAi) response are possible explanations.^{8,42–44} Regardless of the mechanism, infection with ISFs is another example of how a mosquito's microbiome can affect its vector competence for certain arboviruses. The effect of the endosymbiotic bacterium *Wolbachia* on the vector competence of *Aedes aegypti* for dengue virus and several other arboviruses has been well documented.^{45–47} The effect of the midgut microbiota of *Ae. aegypti* mosquitoes on their vector competence for dengue virus have also been demonstrated.^{48,49}

FIGURE 2. Maximum-likelihood (ML) phylogenetic tree of the two novel Mercadeo virus strains (MECDV) and other members of the genus *Flavivirus*. Bootstrap values are shown for most clades. All horizontal branch lengths are drawn to scale (bar = 0.2 nucleotide substitutions per site). The tree is midpoint rooted for purposes of clarity only. See Supplemental Table 1 for virus abbreviations and GenBank accession numbers. NKV = no known vector.

The concept of “vector competence” has been defined “as the innate ability of a vector to acquire a pathogen and to successfully transmit it to another susceptible host.”⁵⁰ It is generally accepted that the vector competence of a hematophagous arthropod is influenced by a number of external (extrinsic) and internal (intrinsic) factors, such as temperature, larval nutrition, age, host preference, genetic make-up, and innate immunity.^{50,51} The available evidence suggests that the insect’s microbiome is yet another variable that affects vector competence. Just as all *Ae. aegypti* or *Culex quinquefasciatus* females in a given field population do not have the same genetic make-up or age, they also probably vary in their microbiomes. We still have much to learn about the various factors affecting vector competence and their interaction, but the microbiome appears to be an important one. This fact should give pause to mathematicians and others who attempt to create predictive models of the outcome of the introduction of an arboviral pathogen into a new geographic region or even to model the course and duration of an ongoing arbovirus outbreak.

Results of our phylogenetic studies (Figure 2) indicate that MECDV is most closely related to CFAV and NAKV. Despite their genetic similarities, the three ISFs have quite different

mosquito associations and geographic distributions. CFAV has been isolated mainly from *Ae. aegypti* and *Ae. albopictus* mosquitoes collected in the United States,³⁹ Mexico,⁵² Puerto Rico,⁵³ Thailand, and Indonesia.⁵⁴ CFAV also have been isolated repeatedly from pools of male as well as female mosquitoes,^{53,54} suggesting that the virus is maintained in nature by vertical transmission. Bolling and others³⁹ described a laboratory colony of *Ae. albopictus*, established from adults collected in Galveston, TX, that was persistently infected with CFAV; the virus was present in most of the mosquitoes of both sexes generation after generation. Less information is available for NAKV or MECDV. A single isolate of NAKV was reported from a pool of *Mansonia africana* mosquitoes collected in Uganda,²⁴ and our two isolates of MECDV were obtained from pools of *Culex* spp. mosquitoes collected in Panama.

CFAV, NAKV, and MECDV all replicate in C6/36 (*Ae. albopictus*) cells, although some strains did not produce obvious CPE on initial culture. In fact, many strains were initially detected by RT-PCR done directly on homogenates of field-collected mosquitoes or on C6/36 culture medium after an initial passage in the mosquito cells.^{24,53,54} The first isolate

(prototype) of CFAV was made from a chronically infected *Ae. aegypti* (Peleg) cell line.⁴ Spent medium from the infected *Ae. aegypti* cells caused extensive syncytium formation when inoculated into a culture of *Ae. albopictus* (C6/36) cells, consequently the agent was named “cell-fusing agent virus” (CFAV).^{4,5} Our experience with field strains of CFAV isolated from mosquitoes collected in the United States and Mexico has been that some strains produce obvious CPE on the initial culture in C6/36 cells, whereas others require two or more serial passages in C6/36 cells before the characteristic CPE (syncytia, cell death, and retarded growth) become apparent. For this reason, RT-PCR is probably a more sensitive assay system for detecting these viruses in field-collected mosquitoes. However, pools of field-collected mosquitoes may contain other types of insect-specific viruses that cause CPE in C6/36 cell cultures and are not detected using flavivirus primers.^{7,55} When deep sequencing is done on pooled mosquito samples or on C6/36 cultures of such samples, it is not uncommon to detect sequences of two or more different types (taxa) of viruses.^{56,57}

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