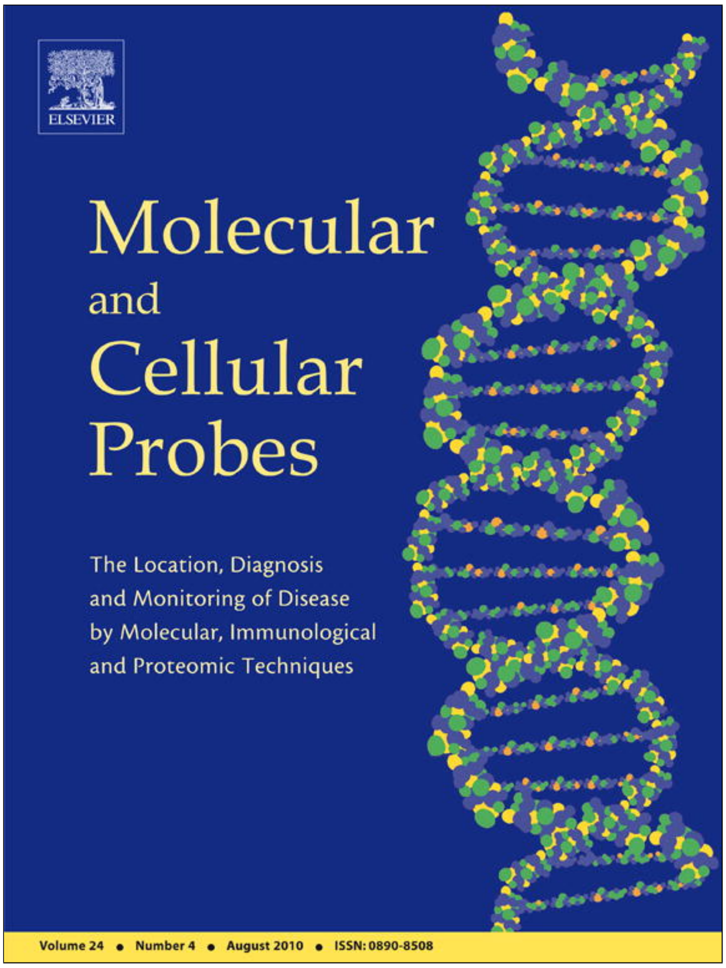
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14. ABSTRACT
Flaviviruses are a highly diverse group of RNA viruses classified within the genus Flavivirus, family Flaviviridae. Most flaviviruses are arthropod-borne, requiring a mosquito or tick vector. Several flaviviruses are highly pathogenic to humans; however, their high genetic diversity and immunological relatedness makes them extremely challenging to diagnose. In this study, we developed and evaluated a broad-range Flavivirus assay designed to detect both tick- and mosquito-borne flaviviruses by using RT-PCR/electrospray ionization mass spectrometry (RT-PCR/ESI-MS) on the Ibis T5000 platform. The assay was evaluated with a panel of 13 different flaviviruses. All samples were correctly identified to the species level. To determine the limit of detection for the mosquito-borne primer sets, serial dilutions of RNA from West Nile virus (WNV) were assayed and could be detected down to an equivalent viral titer of 0.2 plaque-forming units/mL. Analysis of flaviviruses in their natural biological background included testing Aedes aegypti mosquitoes that were laboratory-infected with dengue-1 virus. The assay accurately identified the virus within infected mosquitoes, and we determined the average viral genome per mosquito to be 2.0   106. Using human blood, serum, and urine spiked with WNV and mouse blood and brain tissues from Karshi virus-infected mice, we showed that these clinical matrices did not inhibit the detection of these viruses. Finally, we used the assay to test field-collected Ixodes scapularis ticks collected from sites in New York and Connecticut. We found 16/322 (5% infection rate) ticks positive for deer tick virus, a subtype of Powassan virus. In summary, we developed a single high-throughput Flavivirus assay that could detect multiple tick- and mosquito-borne flaviviruses and thus provides a new analytical tool for their medical diagnosis and epidemiological surveillance.

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Rapid identification of vector-borne flaviviruses by mass spectrometry

Rebecca J. Grant-Klein [a], Carson D. Baldwin [a], Michael J. Turell [a], Cynthia A. Rossi [a], Feng Li [b], Robert Lovari [b], Chris D. Crowder [b], Heather E. Matthews [b], Megan A. Rounds [b], Mark W. Eshoo [b], Lawrence B. Blyn [b], David J. Ecker [b], Rangarajan Sampath [b], Chris A. Whitehouse [a,*]

[a] United States Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, MD 21702, USA
[b] Ibis Biosciences, 1891 Rutherford Rd., Carlsbad, CA 92008, USA

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ABSTRACT

Flaviviruses are a highly diverse group of RNA viruses classified within the genus *Flavivirus*, family *Flaviviridae*. Most flaviviruses are arthropod-borne, requiring a mosquito or tick vector. Several flaviviruses are highly pathogenic to humans; however, their high genetic diversity and immunological relatedness makes them extremely challenging to diagnose. In this study, we developed and evaluated a broad-range *Flavivirus* assay designed to detect both tick- and mosquito-borne flaviviruses by using RT-PCR/electrospray ionization mass spectrometry (RT-PCR/ESI-MS) on the Ibis T5000 platform. The assay was evaluated with a panel of 13 different flaviviruses. All samples were correctly identified to the species level. To determine the limit of detection for the mosquito-borne primer sets, serial dilutions of RNA from West Nile virus (WNV) were assayed and could be detected down to an equivalent viral titer of 0.2 plaque-forming units/mL. Analysis of flaviviruses in their natural biological background included testing *Aedes aegypti* mosquitoes that were laboratory-infected with dengue-1 virus. The assay accurately identified the virus within infected mosquitoes, and we determined the average viral genome per mosquito to be 2.0×10^6. Using human blood, serum, and urine spiked with WNV and mouse blood and brain tissues from Karshi virus-infected mice, we showed that these clinical matrices did not inhibit the detection of these viruses. Finally, we used the assay to test field-collected *Ixodes scapularis* ticks collected from sites in New York and Connecticut. We found 16/322 (5% infection rate) ticks positive for deer tick virus, a subtype of Powassan virus. In summary, we developed a single high-throughput *Flavivirus* assay that could detect multiple tick- and mosquito-borne flaviviruses and thus provides a new analytical tool for their medical diagnosis and epidemiological surveillance.

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1. Introduction

Flaviviruses are single-stranded, positive-sense, RNA viruses classified in the genus *Flavivirus* within the family *Flaviviridae*. There are more than 50 virus species within the genus, and most are arthropod-borne, being transmitted to vertebrates by infected mosquitoes or ticks [16]. Phylogenetic analysis has demonstrated three major groups of flaviviruses, comprising the mosquito-borne, tick-borne, and no-known-vector clades [21]. Of the known flaviviruses, approximately 50% are recognized human pathogens causing fever, encephalitis, or hemorrhagic disease; however, for many of the others, their pathogenic potential has not been well-studied [16]. Important mosquito-borne flaviviruses include dengue viruses serotypes 1–4 (DENV 1–4), yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), and St. Louis encephalitis (SLEV).

Viruses in the tick-borne encephalitis virus (TBEV) complex are significant human pathogens in various parts of Europe and Asia [15] and have been defined geographically and phylogenetically into the European, Far Eastern, and Siberian subtypes [9]. Several tick-borne flaviviruses can also cause hemorrhagic disease. Important examples of these viruses include Omsk hemorrhagic fever virus (OHFV) in Russia, Kyasanur Forest disease virus (KFDV) in India, and the closely related Alkhurma hemorrhagic fever virus (AHFV), which has been a rare cause of hemorrhagic fever in Saudi Arabia since its initial description in 1995 [36]. Powassan virus (POWV) is the only recognized tick-borne flavivirus pathogenic to humans in the Americas [6]. It occurs in parts of eastern Russia, Canada, and in isolated foci in the northeastern and north-central United States. Cases of encephalitic disease caused by this virus

* Corresponding author. Tel.: +1 301 619 2098; fax: +1 301 619 2492.
E-mail address: chris.whitehouse@us.army.mil (C.A. Whitehouse).

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appear to be on the increase in the United States [17]. Deer tick virus (DTV), which is closely related to POWV, was first isolated from *Ixodes scapularis* ticks in 1997 from North America [14,32]. DTV is now considered a genetic subtype of POWV and was recently shown to be a cause of fatal encephalitis [31].

Development of broad-range flavivirus diagnostic assays has been problematic largely because of the high degree of genetic diversity and immunological cross-reactivity among these viruses. Many molecular amplification assays for flaviviruses have been developed over the years [22]. Several attempts to develop broad-range or universal flavivirus detection assays have also been made, typically using RT-PCR with degenerate primers targeted to conserved regions of the genome. However, these assays require either the sequencing of the resulting amplicons [2,24,26] or analysis by restriction digestion of the amplicons [13]. While the use of mass spectrometry as a diagnostic tool has made great strides in recent years [11], it has mostly been used for bacterial identification by the examination of protein or lipid profiles using matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF). In contrast, the Ibis T5000 (Ibis Biosciences, Inc., a subsidiary of Abbott Molecular) analyses DNA and determines the base composition ($A_xG_xT_xC_x$) of PCR amplicons by using electrospray ionization mass spectrometry (ESI-MS) (Fig. 1) [8,28]. In the present study, we developed an 8-primer-pair broad-range flavivirus assay using RT-PCR coupled with ESI-MS. Using this assay, we tested multiple strains of viruses, representing both mosquito-borne and tick-borne flaviviruses. In addition, to show that the assay is capable of detecting viruses in biologically- and clinically-relevant matrices, we examined laboratory-infected mosquitoes, blood and brain tissues from laboratory-infected mice, and virus-spiked human clinical specimens. Furthermore, field-collected ticks from several sites in the U.S. Northeast were tested for the presence of naturally occurring *Flavivirus* infection. Due to the increased geographic distribution and severity of disease caused by members of the *Flavivirus* genus, novel methods for their detection are critical for both vector surveillance efforts and clinical diagnosis.

2. Materials and methods

2.1. Viral isolates, plaque assay, and RNA extraction

The viruses used in this study were part of the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) virus culture collection, and their characteristics are listed in Table 1. RNA from virus cultures and from pooled mosquitoes was extracted using TRIzol-LS® (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. RNA was quantified and checked for quality by measuring absorbance at 260 and 280 nm. Samples were stored at −70 °C until used.

2.2. Tick collections

Adult ticks were collected by flagging from multiple sites in New York and Connecticut. Ticks were homogenized using a combination of large and small yttria-stabilized zirconium oxide beads (Glen Mills, Clifton, NJ) and total nucleic acids were extracted using a modification of the Qiagen Virus MinElute kit (Qiagen, Valencia, CA) as described elsewhere [5].

2.3. Mosquito inoculation and virus plaque assay

Aedes aegypti mosquitoes (Rockefeller strain) were obtained from the Uniformed Services University of Health Sciences and inoculated intrathoracically with 0.3 µL of a virus suspension containing about 10^5 plaque-forming units (PFU)/mL ($10^{1.5}$ PFU inoculated per mosquito) of DENV1-4, or YFV. After inoculation, mosquitoes were held in cardboard containers in an incubator maintained at 26 °C for 7 days and were provided apple slices daily as a carbohydrate source. The mosquitoes were triturated in 0.6 mL of diluent containing 10% heat-inactivated fetal bovine serum in Medium 199 with Earle's salts, 5 µg of amphotericin B, 50 µg of gentamicin, 100 units of penicillin, and 100 µg of streptomycin per mL. An aliquot of 0.1 mL of each suspension was added to 0.9 mL of diluent and frozen at −70 °C until tested for virus by plaque assay.

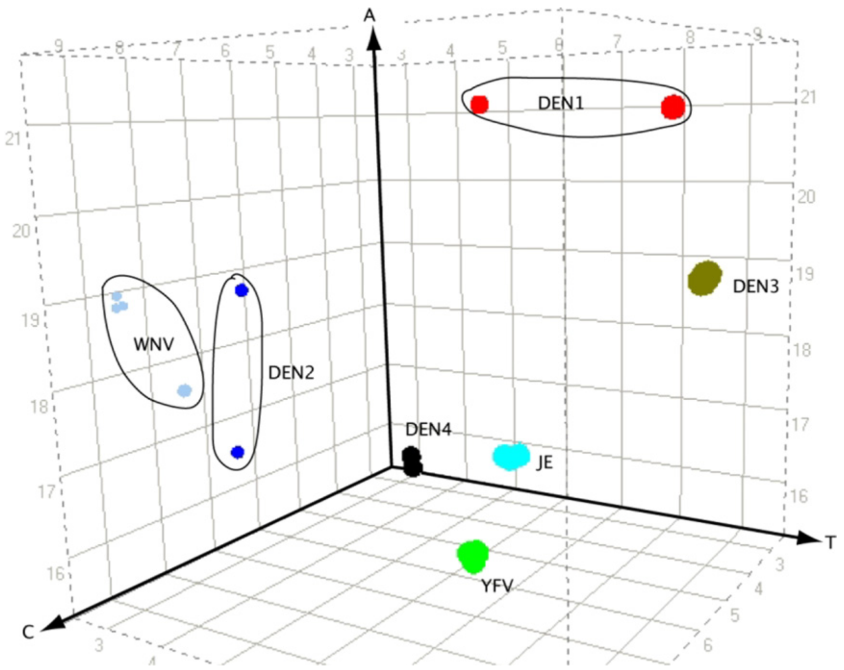


Fig. 1. Theoretical species resolution for a subset of medically important *Flavivirus* sequences in GenBank. Most major species are clearly differentiated from each other. Additional primer pairs designed to specific species groups will provide added resolving power within a cluster (data not shown).

Table 1
Flaviviruses used in this study.

Virus name	Abbreviation	Strain	Origin/location/year of isolation	GenBank accession no.
Mosquito-borne viruses				
Dengue virus 1	DENV-1	Hawaii	Human/Hawaii/1944	EU848545
Dengue virus 2	DENV-2	S16803	Thailand/?	NR
Dengue virus 3	DENV-3	H87	Human/1956	M93130
Dengue virus 4	DENV-4	H241	Human/1956	AY947539
Japanese encephalitis virus	JEV	Nakayama	Human/Japan/1935	EF571853
St. Louis encephalitis virus	SLEV	MSI-7	Sparrow/Mississippi/1975	EU076711
St. Louis encephalitis virus	SLEV	TBH-28	Human/Florida/1962	EU090145
St. Louis encephalitis virus	SLEV	Parton	Human/Missouri/1933	EU084877
Tembusu virus	TMUV	157	Mosquito/1955	NR
West Nile virus	WNV	NY99	Crow/New York/1999	NC_009942
Yellow fever virus	YFV			
Tick-borne viruses				
Central European subtype TBEV[a]	TBEV-CE	Hypr	Human/~1944	EU303231
Central European subtype TBEV	TBEV-CE	Vergina	Goat/1969	NR
Far Eastern subtype TBEV	TBEV-FE	Sofjin	Human/1937	AF013399
Far Eastern subtype TBEV	TBEV-FE	Mass 93	Human/1993	NR
Karshi virus	KV	UZ-2247	Ticks/Uzbekistan/?	NR
Kyasanur Forest disease virus	KFDV	W371	Human/India/1957	AF013385
Langat	LGTV	TP-21	Tick/Malaysia/1956	EU790644
Omsk hemorrhagic fever virus	OHFV	Belangul	Human/1947	NR
Powassan	POWV	LB	Human/Canada/1958	L06436

[a] Tick-borne encephalitis virus group; NR, not recorded.

Before being transported from the biosafety level-3 suite, 1.5 mL of TRIzol-LS® was added to the remaining 0.5 mL of mosquito suspension, and this sample was then split in half to produce two 1-ml aliquots. RNA was extracted from one of these suspensions as described above. The diluted mosquito suspensions were thawed, serial 10-fold dilutions made, and then tested by plaque assay on LLC-MK-2 cell monolayers to determine the amount of infectious virus present. Methods were essentially identical to those described by Gargan et al. [12], except that the second overlay, containing neutral red, was added 5, rather than 4 days after the initial overlay. Plaques were counted the following day, and titers were expressed a $\log_{10}$ PFU per mL of mosquito suspension. For quantitative analysis, infected mosquito pools contained a single-virus-infected mosquito and nine uninfected mosquitoes. For the mixed infected mosquito pools, two infected mosquitoes (each containing a different virus) were combined with eight uninfected mosquitoes.

2.4. Spiking of human clinical specimen with WNV

Purified WNV was diluted to a final concentration of 1×10^5 PFU/mL in human blood, serum, or urine (Bioreclamation, Inc., Liverpool, NY), or phosphate-buffered saline (PBS) control. Duplicate aliquots of 250 μl of each spiked sample were added to 750 μl of TRIzol-LS®, and the RNA was extracted as described above.

2.5. Tissues from Karshi virus-infected mice

Karshi virus-infected tissues were obtained from suckling mice that were inoculated subcutaneously when 2-day-old with a suckling mouse passage of this virus as part of a previous study on the tissue distribution of this virus [34]. The presence of Karshi virus in the samples was previously confirmed by a Karshi virus-specific quantitative real-time PCR assay performed on the Roche LightCycler as described [34].

2.6. Primer design

Eight primer pairs were designed to target the various members of the genus *Flavivirus* (Table 2). The assay was designed to amplify all mosquito- and tick-borne flaviviruses, with four primer pairs (VIR2215, VIR2217, VIR2211, and VIR2216) being *pan-Flavivirus*, one primer pair (VIR2208) targeting all mosquito-borne flaviviruses, one primer pair (VIR2234) targeting all four serotypes of DENV, and two primer pairs (VIR1026 and VIR1028) targeting all strains of WNV (Table 2). The VIR2217 primer pair targets the RNA-dependent RNA polymerase (RdRp, NS5) gene that is conserved across all known flaviviruses. For each primer region, a database of expected base compositions [A G C T] from all known *Flavivirus* sequences in GenBank was generated (data not shown) and used in the identification and classification. All primers used in this study had a thymine nucleotide at the 5′ end in order to minimize the addition of non-template adenosines during amplification using *Taq* polymerase [4].

2.7. One-step RT-PCR

A PerkinElmer Janus robot (Waltham, MA) was used to set-up each one-step RT-PCR reaction. All RT-PCR reactions were performed in 40 μl reaction using 96-well microtiter plates and an Eppendorf® Mastercycler® thermocycler (Eppendorf, Hamburg, Germany). Each RT-PCR reaction buffer consisted of 3.0 U of Ampli *Taq* Gold (Applied Biossystems, Foster City, CA), 20 mM Tris (pH 8.3), 75 mM KCl, 1.5 mM $MgCl_2$, 0.4 M betaine, 200 μM, dATP, 200 μM dCTP, 200 μM dTTP (each dNTP from Bioline USA, Randolph, MA), 200 μM ^{13}C-enriched dGTP (Spectra Stable Isotopes, Columbia, MD), 10 mM dithiothreitol, 100 ng of sonicated poly-A DNA (Sigma Corp, St Louis, MO), and 250 nM of each primer. The following PCR conditions were used to amplify sequences: 60 °C for 5 min, 4 °C for 10 min, 55 °C for 45 min followed by 8 cycles of 95 °C for 30 s, and 48 °C for 30 s, and 72 °C for 30 s followed by 37 cycles of 95 °C for 15 s, 56 °C for 20 s, and 72 °C for 20 s. The RT-PCR cycle ended with a final extension of 72 °C for 2 min followed by a 4 °C hold.

2.8. Internal positive control RNA

To determine the efficiency of the RT-PCR, each reaction contained a synthetic internal positive DNA control (IPC). The IPC was produced by *in vitro* transcription from a T7 promoter present on

Table 2
RT-PCR primers used in the *pan-Flavivirus* ESI-MS assay.

Primer pair name	Gene target	Coverage	Primer coordinates[a]	Orientation	Primer sequence
VIR2215	NS5	*pan-Flavivirus*	8874–8966	F R	5′-TAGCCGAGCCATCTGGTACATGTGG-3′ 5′-TCTCTGGAAAGCCAGTGGTCTTCATT-3′
VIR2217	NS5	*pan-Flavivirus*	8812–8907	F R	5′-TGTGTCTACAACATGATGGGAAAGAGAGA-3′ 5′-TGCTCCCAGCCACATGTACCA-3′
VIR2208	NS5	*pan*-mosquito-borne viruses	8971–9080	F R	5′-TCATTGAGTGGAGTGGAAGGAGAAGG-3′ 5′-TCCCAGCCGGCTGTGTCATC-3′
VIR2234	NS3	*pan*-dengue viruses	5270–5365	F R	5′-TCATGGATGAAGCACATTTCACAGATCC-3′ 5′-TGAAGATCGCAGCTGCCTCTCCCAT-3′
VIR2211	NS5	*pan-Flavivirus*	8885–8964	F R	5′-TCTGGTTCATGTGGCTGGGAGC-3′ 5′-TCTGCCCAGCCAGTGGTCTTCATT-3′
VIR2216	NS5	*pan-Flavivirus*	8865–8951	F R	5′-TGCCAAGGGAAGCAGGGCCAT-3′ 5′-TGGTCTTCATTGAGGAATCCCAGAGC-3′
VIR1026	NS5	WNV	10,135–10,237	F R	5′-TGGATAGAGGAGAATGAATGGATGGAAGAC-3′ 5′-TCAGGCTGCCACACCAGATGTC-3′
VIR1028	NS3	WNV	5696–5783	F R	5′-TCAAGATGGGGAATGAGATTGCCCTT-3′ 5′-TACTCCGTCTCGTACGACTTTCTGTT-3′

[a] Coordinate numbers for West Nile virus (WNV) primers (VIR1026 and VIR1028) are based on WNV strain 956, GenBank accession number NC_001563. All other primer coordinate numbers are based on dengue virus type 2, GenBank accession number NC_001474.

a cloned synthetic DNA template containing the target regions for the eight primer pairs. The IPC was present in each reaction at a pre-determined concentration (100 copies/RT-PCR reaction) and acted as a calibrant to determine the RT-PCR assay's efficiency and provide semi-quantitative information.

2.9. Mass spectrometry and base composition analysis

After PCR, approximately 30 µl of each RT-PCR reaction was bound to a weak anion exchange matrix where a series of wash steps removed salts and excess reaction reagents as previously described [19]. After clean-up, the purified RT-PCR products were eluted from the stationary phase using a volatile buffer. The Bruker Daltonics microToF (Billerica, MA) mass spectrometer (MS) was used for analyzing the purified DNA [18]. Products from each reaction well were individually sprayed into the MS using a LEAP autosampler (LEAP Technologies, Carrboro, NC). Internal mass standards and plasmid calibrants were utilized to reach a mass accuracy of about 5–10 ppm and provided accurate measurements with high-resolution mass spectra for each sample by previously described protocols [18]. Proprietary signal-processing software was used to deconvolute raw data from mass per charge. This molecular mass was then assigned to the amplicon's empirical molecular mass and correlating base composition, which was matched with those in the system's database. Using a number of statistical considerations and multi-primer results, the organisms were identified [25]. Essentially, results are triangulated across primer pairs to determine organism assignments. The processing software, GenX, is designed to run in an automated fashion on multiple PC systems from an input queue. Parallel processing provides an efficient means of increasing throughput; processing times are 15–45 min/plate depending on spectral complexity. As complementary DNA strands are present, this restriction is used to limit the possible choices of base compositions consistent with molecular weight. These base compositions are then used as hypotheses, from which a spectral representation is modeled. At this point, organisms consistent with the primer pair used in PCR amplification are identified from the amplicon database. A joint least-square algorithm is used to correlate potential organism identifications across multiple primers, using a triangulation method. This computation process improves the confidence of correct organism classification and reduces the possibility of false positives [7]. For every RT-PCR reaction well, the signal amplitude of the IPC and the sample were compared and interpreted to give quantitative results.

3. Results

3.1. Detection of flaviviruses with broad-range PCR primer pairs

Flaviviruses are a genetically diversity group of viruses and thus poses a major challenge for broad-range molecular detection assays. The goal of the work described here was to develop an assay that would allow for the broad detection of all the diverse members of this group of viruses, with emphasis on the medically important vector-borne viruses. To accomplish this goal, a large number of primer pairs were designed and tested (data not shown). Of these, eight primer pairs, which target the NS5 and NS3 viral genes, were chosen for the final assay format (Table 2). Fig. 2 shows a multiple sequence alignment of the *pan-Flavivirus* primer pair, VIR2217, against the known sequences of several flaviviruses in GenBank. For each primer region, a database of expected base compositions was generated (data not shown). Several of the isolates used in this study did not have genomic sequences in GenBank and thus base compositions for the target amplicons were determined experimentally.

3.2. Detection of diverse flaviviruses

The 8-primer pair *pan*-flavivirus ESI-MS assay was evaluated for its ability to detect a panel of diverse flaviviruses comprising both mosquito- and tick-borne viruses, which included viral isolates where there was limited sequence data information in GenBank. Isolates with known sequences showed 100% matches to expected base compositions (Fig. 3). Primer pairs VIR2215 and VIR2217 showed the broadest coverage and amplified all isolates (Fig. 3). Base compositions were taken from at least two or more amplicons for the various primer pairs to give species resolution and for distinguishing isolates at the subtype level. We also determined that additional virus passages in culture did not alter the base composition results (see multiple lots of the same virus in Fig. 3).

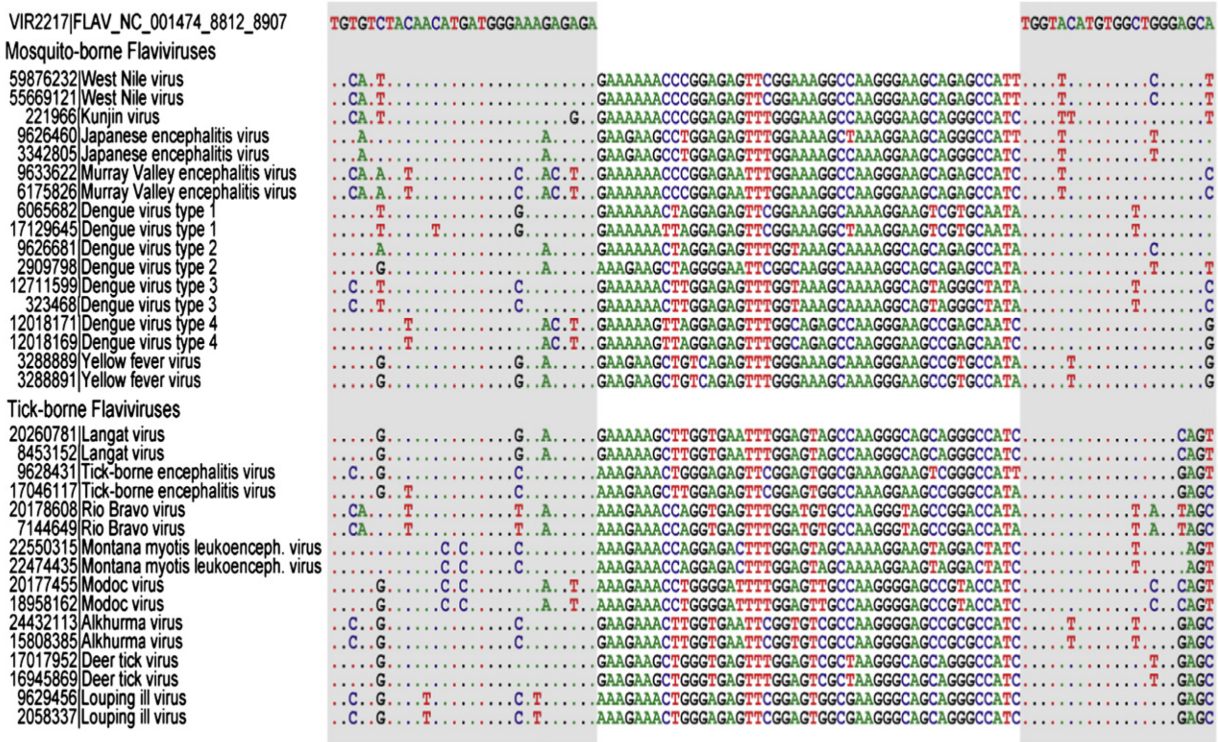


Fig. 2. Multiple sequence alignment of the *Flavivirus* primer pair VIR2217 targeting the NS5 gene and containing over 230 different sequences representing all known flaviviruses. Two representative sequences from each major clade within this genus are shown here. Dots in the column represent identity of each target virus sequence to the primer sequence (top row). Primer pair coordinates shown here are based on the dengue virus 2 (GenBank accession number NC001474).

	Primer Pair [A G C T]							
Organism (Strain)	2217	2234	2215	2216	2208	2211	1028	1026
Central European Encephalitis (Hypr)	[18 12 38 28]	No Prime	[26 20 29 18]	[21 18 30 18]	No Prime	[23 16 27 13]	No Prime	No Prime
Central European Encephalitis (Vergina-R4121T)	[18 12 36 30]	No Prime	[26 21 29 17]	[21 18 30 18]	No Prime	No Prime	No Prime	No Prime
Central European Encephalitis (Vergina-R4121W)	[18 12 36 30]	No Prime	[26 21 29 17]	[21 18 30 18]	No Prime	No Prime	No Prime	No Prime
Central European Encephalitis (Vergina-R4249T)	[18 12 36 30]	No Prime	[26 21 29 17]	[21 18 30 18]	No Prime	No Prime	No Prime	No Prime
Dengue (Hawaii)	[36 32 10 18]	[25 29 25 17]	[21 26 21 25]	No Prime	[37 33 19 21]	[15 26 18 21]	No Prime	No Prime
Japanese Encephalitis (Nakayama-R1664T)	[33 34 13 16]	[28 26 21 20]	[21 28 19 25]	[21 27 17 22]	No Prime	[16 28 14 22]	No Prime	[32 35 15 21]
Japanese Encephalitis (Nakayama-RV029T)	[33 34 13 16]	[28 26 21 20]	[21 28 19 25]	[21 27 17 22]	[36 37 20 17]	[16 28 14 22]	No Prime	[32 35 15 21]
Japanese Encephalitis (Nakayama-RV030T)	[33 34 13 16]	No Prime	[21 28 19 25]	[21 27 17 22]	No Prime	[16 28 14 22]	No Prime	No Prime
Kyasanur Forest Disease (R2062)	[20 16 34 26]	No Prime	[24 20 29 20]	[20 17 30 20]	No Prime	[21 16 27 15]	No Prime	No Prime
Langat (Yaru)	[19 14 33 28]	No Prime	[24 24 28 17]]	[20 19 30 18]	No Prime	No Prime	No Prime	No Prime
Omsk (Balangul)	[17 14 36 29]	No Prime	[24 22 29 18]	No Prime	No Prime	[22 17 27 13]	No Prime	No Prime
Powassan (R2080T)	[29 35 13 19]	No Prime	[18 26 21 28]	[19 27 20 21]	No Prime	[43 58 30 40]	No Prime	No Prime
Russian Spring-Summer Encephalitis (Sophy)	[30 35 13 18]	No Prime	[19 27 20 27]	[14 30 17 26]	No Prime	[13 26 16 24]	No Prime	No Prime
Russian Spring-Summer Encephalitis (Mass 93)	[14 14 34 34]	No Prime	[27 22 21 22]	[22 19 27 19]	No Prime	[24 17 24 14]	No Prime	No Prime
St. Louis Encephalitis (MSI-7)	[33 34 12 17]	[29 24 20 22]	[19 28 19 27]	[19 29 16 23]	No Prime	[14 27 15 24]	[30 25 12 18]	[35 33 17 18]
St. Louis Encephalitis (TBH-28)	[33 34 12 17]	[28 25 20 22]	[18 29 22 24]	[19 29 16 23]	No Prime	[14 27 17 22]	No Prime	[35 33 17 18]
St. Louis Encephalitis (Porton)	[33 34 12 17]	[29 24 19 23]	[19 28 22 24]	[19 29 17 22]	No Prime	[14 27 18 21]	No Prime	[36 32 17 18]
Tembusu (157)	[31 36 11 18]	[19 22 25 29]	[27 18 29 19]	No Prime	No Prime	[24 13 28 15]	No Prime	[18 16 28 41]
West Nile Virus (R4258T)	[15 15 33 33]	No Prime	[26 23 27 17]	[22 20 28 17]	No Prime	[24 17 27 12]	[18 15 25 30]	[17 16 34 36]
West Nile Virus (R4260T)	[15 15 33 33]	No Prime	[26 23 27 17]	[22 20 28 17]	No Prime	[24 17 27 12]	[18 15 25 30]	[17 16 34 36]
West Nile Virus (RS#2T)	[15 15 33 33]	No Prime	[26 23 27 17]	[22 20 28 17]	No Prime	[24 17 27 12]	[18 15 25 30]	[17 16 34 36]
West Nile Virus (R4272T)	[15 15 33 33]	No Prime	[26 23 27 17]	[22 20 28 17]	No Prime	[24 17 27 12]	[18 15 25 30]	[17 16 34 36]

Fig. 3. Base composition [A G C T] data of the RT-PCR amplicons generated by the pan-*Flavivirus* ESI-MS assay. Identical base compositions within a column are the same color. Unique base compositions are shown with white backgrounds. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

As evidence of the assay's ability to detect mixtures of viruses in a sample, a presumably pure laboratory preparation of OHFV was tested and found to be contaminated with YFV. Examining the base composition for four different gene target amplicons (VIR2216 only generated a base composition for YFV) and the mass spectra generated for the forward and reverse DNA strands of the amplicons, it was clear that there were two distinct DNA signatures, which were identified as OHFV and YFV by the *Flavivirus* RT-PCR/ESI-MS assay (Fig. 4).

3.3. Sensitivity of the broad-range PCR primer pairs

To determine the sensitivity of the mosquito-borne primer pairs, WNV was serially diluted 2-fold from a known titer and tested in replicates of 10. All six mosquito-borne primer sets (VIR2215, VIR2217, VIR2211, VIR2216, VIR1026, VIR1028) had 100% sensitivity (10/10 reactions were positive) at 25 PFU/mL and all, except VIR2217, were 100% sensitive down to 1.6 PFU/mL (Fig. 5). Overall, the *Flavivirus* RT-PCR/ESI-MS assay could accurately identify WNV down to 0.2 PFU/mL with VIR2216 having 50% sensitivity, VIR2211 having 20% sensitivity, and VIR2215, VIR1026, VIR1028 having 10% sensitivity (Fig. 5).

3.4. Testing of WNV spiked clinical matrices

To test the assay's ability to detect and correctly identify a flavivirus within relevant human clinical matrices, WNV was spiked into specimens of human blood, urine, and serum and tested with the PCR/ESI-MS assay. The six mosquito-borne *Flavivirus* primer sets had 100% agreement for detecting and identifying WNV from all three of the clinical matrices, which included blood, urine, serum, and a PBS control. Each of the base compositions for each primer set were 100% identical for the four different sample backgrounds (Table 3).

3.5. Identification of Karshi virus from laboratory-infected mice

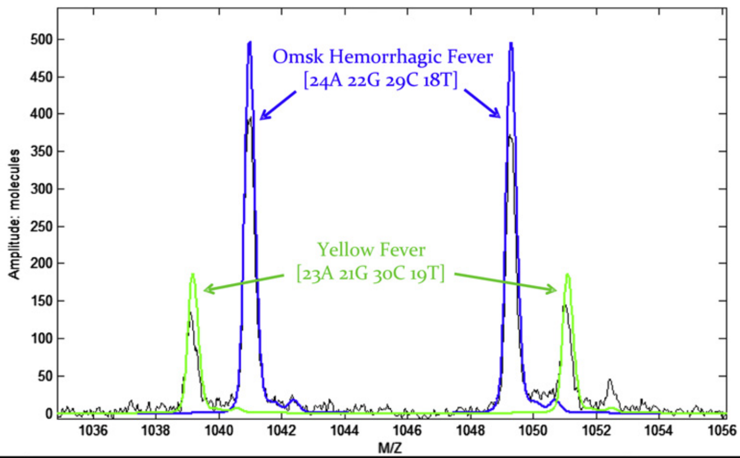
To further evaluate the assay's performance with *Flavivirus*-infected mammalian tissues, Karshi virus-infected blood and brain tissues were obtained from suckling mice that were inoculated subcutaneously when 2-days-old with virus as part of a previous study on the tissue distribution of this virus. RT-PCR/ESI-MS analysis clearly detected Karshi virus in both blood and brain tissues and confirmed high levels of this neurotropic virus in the brain of infected mice (Table 4) as was shown previously using a Karshi virus-specific quantitative real-time RT-PCR assay [34].

3.6. Detection of flaviviruses from laboratory-infected mosquitoes

The ability of the RT-PCR/ESI-MS assay to detect *Flavivirus* RNA from infected mosquitoes was tested by using a blinded set of laboratory-infected mosquitoes and uninfected controls. From 16 different mosquito pools, the correct virus was identified in 15 of the samples (94% correct), highlighting the assay's ability to distinguish between closely related DENV serotypes (Table 5). However, in one sample (W123) only a DENV 3-infected mosquito was present in the sample, and the system also identified DENV 2. And another sample (W131) was a mixed pool containing both DENV 1 and DENV 2; however, the system only detected DENV 2. The one sample that was completely incorrect (T136) was a negative control containing only uninfected mosquitoes and the system identified it as containing WNV. No WNV was used to infect any of the mosquitoes; however, we cannot rule out possible contamination of the sample with WNV RNA or PCR amplicon.

3.7. Quantification of DENV viral load in individual infected mosquitoes

Each reaction well in the RT-PCR/ESI-MS assay contained an IPC to allow for quantitative analysis. Thus, we wanted to test the quantitative ability of the assay to determine the viral load (based on genome equivalents) of DENV 1 present in individual virus-infected mosquitoes and compare that to plaque titer of the same mosquitoes. Ten mosquito pools consisting of one DENV 1-infected and nine uninfected mosquitoes were tested in duplicate using the RT-PCR/ESI-MS assay and virus plaque assay. The mean number of genome equivalents per mosquito was determined to be 2.0×10^6,



Stock ID	Primer Pair 2217	2234	2215	2216	2208	2211	1028	1026	System ID
Flavi007-Omsk	[17 14 36 29]	No Prime	[24 22 29 18]	No Prime	No Prime	[22 17 27 13]	No Prime	No Prime	Omsk Hemorrhagic Fever
	[19 17 35 25]	No Prime	[23 21 30 19]	[20 17 30 20]	No Prime	[21 16 27 15]	No Prime	No Prime	Yellow Fever

Fig. 4. Mass spectra of RT-PCR amplicons derived from an Omsk hemorrhagic fever virus (OHFV) stock culture contaminated with YFV. Labels and signals are colored according to the theoretical spectra for each organism: blue = OHFV, green = YFV. Actual spectra generated are traced in black and correspond to the sense and antisense DNA strands. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

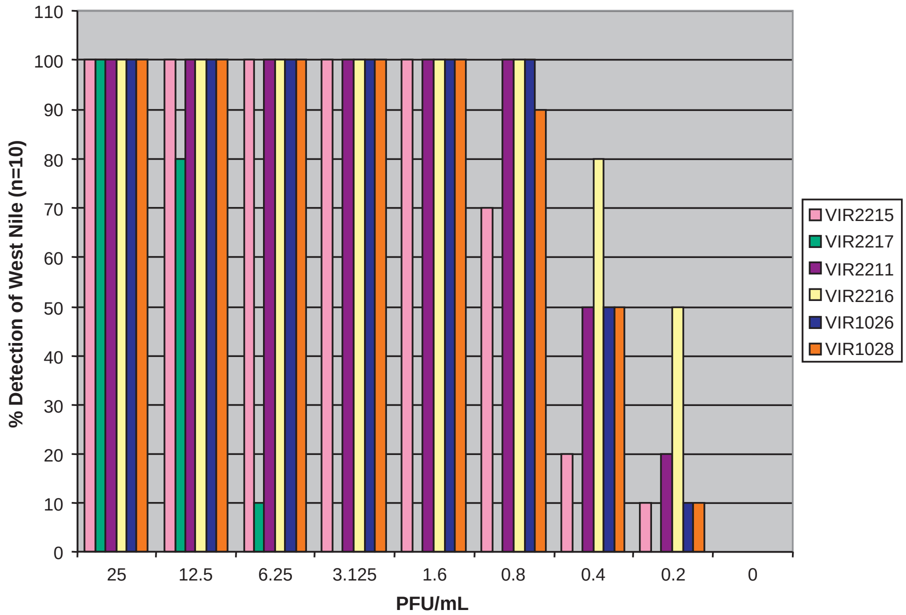


Fig. 5. Sensitivity of the six mosquito-borne *Flavivirus* RT-PCR primer pairs. From a known West Nile virus (WNV) titer, the RNA was serially diluted ten-fold. Ten replicates were performed to determine the sensitivity of each dilution with a score of 100% indicating that all reactions clearly identified WNV.

and the mean plaque titer was approximately 100-fold less at 1.3×10^4 PFU per mosquito (Fig. 6).

3.8. Screening of field-collected ticks for Powassan virus

The *pan-Flavivirus* primer pairs, VIR2215 and VIR2217, were used to screen field-collected *I. scapularis* ticks collected from multiple sites in New York and Connecticut during the 2008 and 2009 tick seasons (Table 6). We found 16 ticks out of a total of 322 tested (5% infection rate) positive for POWV and many of these positive ticks were further characterized as having deer tick virus, a recognized subtype of POWV. Distinct signals can be seen for both the forward and reverse strands of amplicon DNA obtained from specimen G110708MR-5 using the *pan-Flavivirus* primer sets VIR2215 and VIR2217 (Fig. 7). Of note, the assay was able to distinguish three different POWV signatures based on base composition data (Table 6). Approximately 700 nucleotides of viral RNA from specimens G110708MR-5 and G082908CP-11 were sequenced using the primers of Telford et al. [32]. The resulting sequences showed 97% identity to DTV, but only 87% identity to POWV (data not shown) indicating that the *Flavivirus* found was the DTV subtype of POWV. Only a single tick was found to have a third unique RT-PCR/ESI-MS base composition signature (specimen #082908CP-11), but we were unable to obtain sequence information to confirm the virus subtype due to limited sample volume.

4. Discussion

With over 70 different viral species classified in the genus *Flavivirus*, broad-range detection of these viruses has been extremely problematic. Detection and identification of flaviviruses is made even more complicated by the fact that these viruses are RNA viruses, which evolve rapidly, and therefore, subtle changes in their genomes can rapidly make a once highly sensitive and specific molecular based assay obsolete. In the current study, we have developed a broad-range (i.e., *pan-Flavivirus*) RT-PCR/ESI-MS assay that could rapidly identify and discriminate multiple species of vector-borne flaviviruses in a single high-throughput assay. This assay, performed on the Ibis T5000 system, uses standard one-step RT-PCR with broad-range primers targeting the breadth of the genus *Flavivirus*, followed by base composition determination using a time-of-flight mass spectrometer. In this study, we showed that the RT-PCR/ESI-MS assay accurately detected and identified a wide range of flaviviruses including multiple tick- and mosquito-borne viruses (Fig. 3). Using WNV, the sensitivity of the assay was determined to be approximately 2 PFU/mL; however, detection was still possible with some of the primer sets, albeit at lower sensitivities, down to 0.2 PFU/mL (Fig. 5). This limit of detection is consistent with those reported for various traditional and real-time RT-PCR assays for WNV and other flaviviruses [22,23] and other mosquito-borne arboviruses [35]. It is also consistent with the sensitivity of a previously developed RT-PCR/ESI-MS assay for the

Table 3
West Nile virus (WNV) was spiked into different human clinical matrices at a known concentration of 1×10^5 PFU per mL and tested with the PCR/ESI-MS assay. Corresponding base composition data are shown for each of the mosquito-borne virus primers used in the assay. Calculated values for the mean $\log_{10}$ genome equivalents per mL for each specimen type are also given.

Sample matrix	GE[a]	Primer pairs [A G C T]					
		2217	2215	2216	2211	1028	1026
Blood	3.8	[15 15 33 33]	[26 23 27 17]	[22 20 28 17]	[24 17 27 12]	[18 15 25 30]	[17 16 34 36]
Serum	3.1	[15 15 33 33]	[26 23 27 17]	[22 20 28 17]	[24 17 27 12]	[18 15 25 30]	[17 16 34 36]
Urine	3.7	[15 15 33 33]	[26 23 27 17]	[22 20 28 17]	[24 17 27 12]	[18 15 25 30]	[17 16 34 36]
PBS	3.5	[15 15 33 33]	[26 23 27 17]	[22 20 28 17]	[24 17 27 12]	[18 15 25 30]	[17 16 34 36]

[a] Mean $\log_{10}$ genome equivalents per mL.

Table 4
Comparison of a Karshi virus-specific quantitative real-time RT-PCR assay[a] and the *pan-Flavivirus* RT-PCR/ESI-MS assay for testing of blood and brain tissues from experimentally-infected mice.

Mouse ID	Tissue type	Days post-infection	Quantitative real-time RT-PCR (GE[b])	*pan-Flavivirus* RT-PCR/ESI-MS	
				System ID	GE[b]
102	Blood	1	5.3	Pos-Karshi virus	5.8
114	Brain	5	9.2	Pos-Karshi virus	ND[c]
119	Brain	6	11.0	Pos-Karshi virus	ND
134	Brain	9	10.3	Pos-Karshi virus	ND

[a] The quantitative real-time RT-PCR assay used here was developed and published previously [34].
[b] Mean $\log_{10}$ genome equivalents per mL of blood or g of tissue.
[c] Not determined due to the high genome equivalents present in the brain tissue were above the linearity for quantitative analysis of the ESI-MS assay.

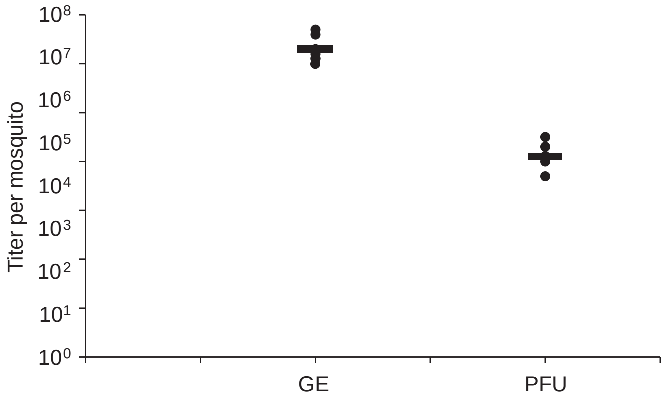


Fig. 6. Viral load per individual mosquito as estimated by the quantitative *pan-Flavivirus* ESI-MS assay and plaque assay. Seven *Aedes aegypti* mosquitoes were inoculated intrathoracically inoculation with approximately $10^{1.5}$ PFU of DENV-1. Seven days after inoculation, mosquitoes were processed as described in the Materials and methods and were tested. Horizontal line indicates mean titer. GE, genome equivalents; PFU.

alphaviruses (assuming that 30 genome equivalents is approximately equal to 3 PFU) [10].

One benefit of the *pan-Flavivirus* RT-PCR/ESI-MS assay is its ability to identify more than one type of virus in a sample. This was evident while we were using the assay to perform quality control of our purified virus stocks for our culture collection. For example, one sample of OHFV was clearly contaminated with YFV (Fig. 4). This characteristic of the assay would also have clear benefits for use in vector surveillance where two or more flaviviruses are co-circulating within the same geographic region, as is the case for WNV and DENV or for multiple serotypes of DENV for example. Because mosquitoes are often tested in pools of up to 50 or even 100, it would be very possible to have more than one-infected mosquito within any given pool. Likewise, individuals living in geographic regions were multiple arboviruses co-circulate, are at risk of co-infections, which has been documented previously [29,30].

We further demonstrated the quantitative nature of the assay by determining of the average number of viral genomes of DENV 1 per individual laboratory-infected *Ae. aegypti* mosquito. The average viral load per infected mosquito was calculated to be 2.0×10^6 genomes or 1.3×10^4 PFU (Fig. 6), which is consistent with previous studies [1,20]. Furthermore, our finding that the number of genomes per individual infected mosquito was about 100-fold higher than the plaque titer is consistent with other studies showing that PFU was consistently lower than RNA copy number by 2–3 $\log_{10}$ for both cell culture and mosquitoes infected with DENV [27].

Though it was important to validate the assay with well-characterized samples that have been tested on other diagnostic platforms, the detection of viruses such as Tembusu and Langat viruses demonstrates the assay's ability to identify less known flaviviruses where little or no sequence data are available. This attribute of the assay will be particularly useful in detecting rapidly evolving RNA viruses, or those that are completely novel, especially from field-collected specimens. The high-throughput and broad nature of the Ibis T5000 platform makes it highly amendable to public health laboratory surveillance work. In particular, the previously developed *pan-Alphavirus* ESI-MS assay could be combined with the *pan-Flavivirus* assay developed in this study into a single "Arbovirus kit" and used for mosquito and/or tick-borne arbovirus surveillance. To demonstrate the usefulness of the *Flavivirus* assay in vector-borne pathogen surveillance, we screened 322 field-collected *I. scapularis* ticks collected from New York and Connecticut for the presence of flaviviruses. While several medically important tick-borne *Flavivirus* are known to occur and cause severe neurological disease in Europe and Asia (e.g. tick-borne encephalitis virus), POWV is the only recognized tick-borne *Flavivirus* in the U.S. [6,14]. This is consistent with the fact that DTV, a subtype of POWV, was the only *Flavivirus* found among the ticks tested. Furthermore, we

Table 5
Assay performance for detection of flaviviruses from a blinded panel of laboratory-infected mosquitoes. Base composition data are shown for each of the primer sets in the *Flavivirus* RT-PCR/ESI-MS assay, along with the system identification. Each sample was assayed in triplicate.

Coded Sample ID	Actual Sample ID	Primer pairs Base composition [A G C T]							
		2217	2215	2216	2211	1026	2234	2208	System ID
W121	DENV 1	[36 32 10 18]	ND[a]	ND	[16 25 17 22]	ND	[26 28 24 18]	[37 33 19 21]	DENV 1
W122	DENV 2	[33 34 13 16]	[22 24 23 24]	[21 26 19 21]	[17 24 18 21]	[38 33 14 18]	[30 26 21 19]	[34 40 17 19]	DENV 2
W123	DENV 3	[34 32 10 20]	[22 24 23 24]	[21 26 19 21]	[15 27 19 19]	ND	[28 27 20 21]	[32 36 20 22]	DENV 2, 3
W124	DENV 4	[30 34 13 17]	[19 29 19 26]	[20 29 18 20]	[14 28 14 24]	ND	[26 27 21 22]	[34 38 15 23]	DENV 4
W125	NC[b]	ND	ND	ND	ND	ND	ND	ND	Neg.
W126	DENV 1	[36 32 10 18]	[21 26 21 25]	ND	[16 25 17 22]	ND	[26 28 24 18]	[37 33 19 21]	DENV 1
W127	DENV 2	[33 34 13 16]	[22 24 23 24]	[21 26 19 21]	[17 24 18 21]	ND	[30 26 21 19]	[34 40 17 19]	DENV 2
W128	DENV 3	[34 32 10 20]	[20 28 23 22]	ND	[15 27 19 19]	ND	[28 27 20 21]	[32 36 20 22]	DENV 3
W129	DENV 4	[32 34 12 18]	[19 29 19 26]	ND	[14 28 14 24]	ND	[26 27 21 22]	[34 38 15 23]	DENV 4
W130	NC	ND	ND	ND	ND	ND	ND	ND	Neg.
W131	DENV 1, 2	[33 34 13 16]	[22 24 23 24]	[21 26 19 21]	[17 24 18 21]	[38 33 14 18]	[30 26 21 19]	[37 33 19 21]	DENV 2
T135	YFV	[30 33 13 18]	[19 30 21 23]	[20 30 16 21]	[14 29 16 21]	ND	ND	ND	YFV
T136	NC	[28 30 18 18]	ND	[17 28 20 22]	[12 27 17 24]	[36 34 16 17]	ND	ND	WNV
T137	DENV 1	[36 32 10 18]	[19 30 21 23]	[20 30 16 21]	[16 25 17 22]	ND	[26 28 24 18]	[37 33 19 21]	DENV 1
T138	DENV 1	[36 32 10 18]	[21 26 21 25]	[20 30 16 21]	[16 25 17 22]	ND	[26 28 24 18]	[37 33 19 21]	DENV 1
315–9	NC	ND	ND	ND	ND	ND	ND	ND	Neg.

[a] No detection.
[b] Negative control.

Table 6
Characterization of field-collected *Ixodes scapularis* ticks analyzed with the *pan-Flavivirus* ESI-MS assay.

Tick ID	Collection location	ESI-MS result	Genome copy no.	Base composition [AGCT]	
				VIR2215	VIR2217
120208MR-3	Bridgeport, CT	DTV	200	19 25 23 26	27 36 14 19
120308MR-9	Bridgeport, CT	DTV	300	19 25 23 26	27 36 14 19
082908CP-11	Connetquot, NY	POWV[a]	>330	19 25 22 27	26 37 14 19
082608CP-33	Shelter Island, NY	DTV	>330	19 25 22 27	27 36 14 19
082708CP-5	Shelter Island, NY	DTV	>330	19 25 22 27	27 36 14 19
110608MR-6	Westchester county, NY	DTV	>330	19 25 22 27	27 36 14 19
110608MR-8	Westchester county, NY	DTV	>330	19 25 22 27	27 36 14 19
110608MR-9	Westchester county, NY	POWV[a]	12	ND[b]	27 36 14 19
110708MR-2	Westchester county, NY	POWV[a]	23	ND	27 36 14 19
110708MR-4	Westchester county, NY	POWV[a]	43	ND	27 36 14 19
110708MR-5	Westchester county, NY	DTV	>330	19 25 22 27	27 36 14 19
110708MR-6	Westchester county, NY	POWV[a]	34	ND	27 36 14 19
110708MR-14	Westchester county, NY	POWV[a]	>330	ND	27 36 14 19
110708MR-15	Westchester county, NY	POWV[a]	108	ND	27 36 14 19
052008CC-22	Bridgeport, CT	DTV	79	19 25 22 27	27 36 14 19
061009MR-1	Shelter Island, NY	POWV[a]	13	ND	27 36 14 19

[a] Due to limited specimen DNA extract volume, the POWV subtype was not determined.
[b] No detection.

determined a 5% infection rate among the *I. scapularis* tested from the selected collection sites in New York and Connecticut. Previously published tick infection rates were much lower than our findings. For instance, in the original publication describing DTV from New England in 1995, they found only a 0.4% infection rate among 465 *I. scapularis* ticks collected from Connecticut and Massachusetts [32]. More recent data from a northern Wisconsin focus shows a 1.3% infection rate among 1335 ticks tested [3]. In a 2009 survey of *I. scapularis* collected from several locations surrounding New York City, Tokarz et al. found a 2.0% infection rate for Powassan virus using a Mass Tag PCR approach [33]. Although more studies are needed, our data suggest that the prevalence Powassan virus, and particularly the DTV subtype, may be increasing in the U.S. northeast, and this virus may emerge as an important public health concern in the future.

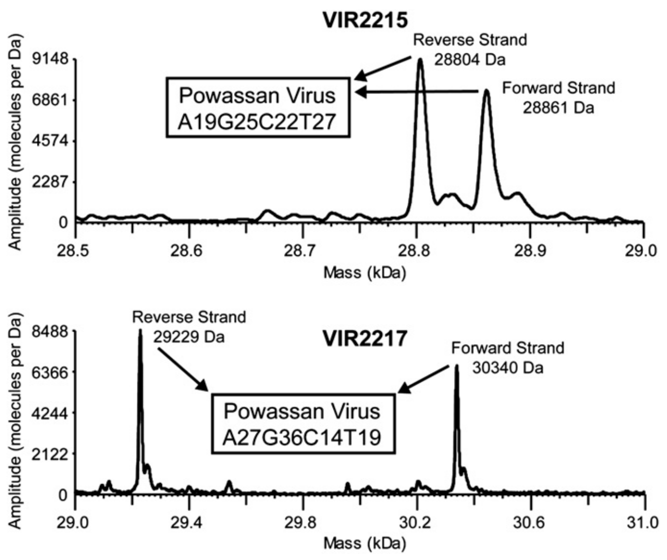


Fig. 7. Mass spectra from primer pairs VIR2215 and VIR2217 showing the sense and antisense DNA strands from a deer tick virus (POWV)-positive field-collected *I. scapularis* tick.

In conclusion, the ability of the *pan-Flavivirus* RT-PCR/ESI-MS assay to rapidly and sensitively identify known and emerging flaviviruses is critical for disease surveillance and for advancing the molecular diagnostic field past single-virus detection assays. We have shown that this assay has the ability to be a broad-range detection tool for known and rare flaviviral species that cause human disease and could benefit clinical diagnostics or studies on the ecology and epidemiology of this important group of viruses.

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