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Development of Conventional and Real-Time Reverse Transcription Polymerase Chain Reaction Assays to Detect Tembusu Virus in *Culex tarsalis* Mosquitoes

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Abstract. Tembusu virus (TMUV) is an important emerging arthropod-borne virus that may cause encephalitis in humans and has been isolated in regions of southeast Asia, including Malaysia, Thailand, and China. Currently, detection and identification of TMUV are limited to research laboratories, because quantitative rapid diagnostic assays for the virus do not exist. We describe the development of sensitive and specific conventional and real-time quantitative reverse transcription polymerase chain reaction assays for detecting TMUV RNA in infected cell culture supernatant and *Culex tarsalis* mosquitoes. We used this assay to document the replication of TMUV in *Cx. tarsalis*, where titers increased 1,000-fold 5 days after inoculation. These assays resulted in the detection of virus-specific RNA in the presence of copurified mosquito nucleic acids. The use of these rapid diagnostic assays may have future applications for field pathogen surveillance and may assist in early detection, diagnosis, and control of the associated arthropod-borne pathogens.

INTRODUCTION

Throughout history, arthropod-transmitted pathogens have been an important cause of human disease. Members of the genus *Flavivirus* (family *Flaviviridae*), including Japanese encephalitis virus (JEV), West Nile (WNV), yellow fever (YFV), and dengue (DENV) viruses, are responsible for millions of clinical cases and tens of thousands of human deaths annually.[1,2] Tembusu virus (TMUV; genus *Flavivirus*, family *Flaviviridae*), a member of the Ntaya serocomplex, was first isolated in 1955 from *Culex tritaeniorhynchus* mosquitoes in Kuala Lumpur, Malaysia,[3] and it is responsible for severe disease in ducks in China.[4] To date, little is known about TMUV; however, several recent reports indicate that TMUV and TMUV-like viruses have also been isolated in other regions of southeast Asia, including Thailand and China.[5–13] Like other flaviviruses, TMUV is a linear, positive-sense, single-stranded RNA virus with a genome size of approximately 11 kb. Its RNA encodes 10 proteins, including three structural proteins—the capsid (C) protein, the membrane (M) protein, and the envelope (E) glycoprotein—and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5).[14]

Current methods of detection and identification of TMUV include cell culture, enzyme immunoassay (EIA), reverse transcription polymerase chain reaction (RT-PCR), and nucleotide sequencing-based assays.[15–18] To protect individuals in any given region of the world from the threat of vector-borne pathogens, we need to be able to rapidly and accurately identify arthropod-borne pathogens, and therefore, appropriate preventive measures can be taken to prevent morbidity and mortality associated with infection with these pathogens. Development of field-based real-time quantitative RT-PCR (qRT-PCR) assays for rapid detection of virus-infected mosquitoes would be a great assistance to preventive medicine and healthcare providers.

A major challenge in analyzing field-collected mosquitoes is that virus infection rates are often very low and require testing hundreds, if not thousands, of mosquito pools, each containing 25–50 mosquitoes. Therefore, qRT-PCR assays for the detection of viral RNA require specific primers and probes that can detect small amounts of viral RNA in the presence of significantly greater amounts of mosquito nucleic acids. In this study, we developed and evaluated specific conventional and qRT-PCR assays for detecting TMUV in infected cell culture supernatant and laboratory-infected mosquitoes.

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MATERIALS AND METHODS

Mosquito processing, RNA extraction, and cDNA synthesis. Mosquitoes collected during a field study in two west central Thailand provinces (Lopburi and Kamphaeng Phet) from March 6 to March 18, 2011 were processed for virus detection as previously described.[19] Briefly, pools containing 1–25 mosquitoes were placed in 1.5-mL microcentrifuge tubes containing one 4.5-mm copper bead and 0.75 mL cell culture media (Eagle's Minimum Essential Medium containing 10% heat-inactivated fetal bovine serum, 0.075% $NaHCO_3$, 50 μg gentamicin, 100 units penicillin, and 100 μg streptomycin/mL). Tubes were vortexed for 5 minutes and then clarified by centrifugation (12,000 × *g* for 10 minutes at 4°C). We added 250 μL supernatant to a 1.5-mL microcentrifuge tube containing 750 μL TRIzol LS (Invitrogen, Carlsbad, CA) for RNA extraction, and the remainder of the supernatant was stored on dry ice or frozen at −70°C.

RNA extraction. RNA was extracted as previously described,[19] except that 1 μL GlycoBlue (Ambion Applied Biosystems, Austin, TX) was used during the RNA precipitation step. The RNA pellet was resuspended in 12 μL nuclease-free water and used immediately or stored at −70°C. First-strand cDNA synthesis was performed as previously described,[19] except that 200 units Superscript III reverse transcriptase (Invitrogen) were used with 10 μL template RNA and 2 μL 50 ng/μL random hexamer.

RT-PCR amplification and gel electrophoresis. Preliminary screening for flavivirus or TMUV nucleic acid, which was adapted from the work by O'Guinn and others,[19] was performed by using puRe TaqReady-To-Go PCR beads (Amersham Biosciences, Piscataway, NJ) in a final volume of 25 μL containing 1–2 μL cDNA and 10 pmol each MA

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Table 1
Conventional and real-time qRT-PCR primers and probe for detecting TMUV

Primer name	Sequence (5′ → 3′)	Genomic position*
Conventional PCR primers (product = 439 bp)		
TMUVE1Fwd	CGTGGCAAAACAGAGAGTCA	E (1556-1575)
TMUVE1Rev	GGAGGTTCCACCTCTACCATAATC	E (1971-1994)
qRT-PCR primers and probe (product = 82 bp)		
TMUV-NS5F	GAGGCGTTGAAGGACTTGGA	NS5 (9080-9099)
TMUV-NS5R	GCGTACATGAGTCCTCCTTCTTT	NS5 (9139-9161)
TMUV-NS5P	6FAM-ACGCAGCACATACCC-MGBNFQ	NS5 (9112-9126)

*Position based on Tembusu (MM1775) GenBank accession number JX477685.

(forward) and cFD2 (reverse) primers[20] or TMUVE1 forward/E1 reverse primers Table 1. Conventional PCR amplification was performed as follows: 95°C for 2 minutes, 35 cycles at 95°C for 30 seconds, 50°C (56°C for TMUV studies) for 30 seconds, and 72°C for 45 seconds followed by 72°C for 7 minutes. Agarose gel electrophoresis of the PCR products and DNA molecular weight marker was performed using 2% E-gel gels (Invitrogen) containing ethidium bromide. After electrophoresis, the PCR product bands were visualized using a transilluminator and recorded using a Polaroid camera (Polaroid, Waltham, MA) and 667 film or a BioRad Gel Doc (BioRad Life Sciences, Hercules, CA).

Viruses. Virus specimens were propagated in primary duck embryo, C6/36 (*Aedes albopictus*), or Vero (African green monkey kidney) cells to produce stock viruses for these studies. Cell culture supernatants were harvested, aliquoted, and stored at −70°C. For those viruses that produced plaques, viral titer was determined by standard plaque assay on Vero cells.

Development of Tembusu-specific conventional PCR primers. Virus sequences, representing laboratory and field isolates, were aligned using the MegaAlign program (Lasergene analysis software; DNASTAR, Madison, WI) and compared with TMUV GenBank sequences (accession nos. AB110493, AB110495, and AF013409). After alignment of genomic sequences, the consensus sequence was imported into the LaserGene PrimerSelect program (Lasergene analysis software; DNASTAR), and selection of primers was determined based on the most ideal characteristics. The sequences of the TMUVE1 forward or TMUVE1 reverse primers and the base pair products of primer combinations are shown in Table 1 and Figure 1.

Development of Tembusu-specific real-time primers and probes. The TMUV NS5 gene was submitted to ABI Custom Assay Services (Foster City, CA) for primer and probe determination. The sequences of the TMUV-NS5 primers and probe determined by this approach are shown in Table 1 and Figure 1. A basic local alignment search tool (BLAST) search was used to conduct a preliminary assessment of oligonucleotide specificity.

One-step qRT-PCRs. One-step qRT-PCRs were performed using the SuperScript III one-step RT-PCR system with platinum *taq* (Invitrogen) in a final volume of 20 μL containing 5 μL RNA, 10 μL 2× reaction buffer, additional 3 mM $MgSO_4$, 5 μg bovine serum albumin (Roche, Pleasanton, CA), 1 μL each 18 μM forward and reverse primers, and 1 μL 5 μM probe. The above concentration of $MgSO_4$ was determined to be optimal for this assay. The qRT-PCR assays were carried out using the Ruggedized Advanced Pathogen Identification Device (RAPID; Idaho Technology, Salt Lake City, UT) and the following cycling conditions: reverse transcription at 50°C for 15 minutes, denaturation at 95°C for 2 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute. The gain was set at four or eight for the TMUV-NS5 primer/probe set.

Mosquito infection. *Cx. tarsalis* mosquitoes were reared in an insectary at 27°C and exposed to a light:dark cycle of 16:8 hours. Adult female mosquitoes were inoculated intrathoracically[21] 6–8 days after emergence with 0.3 μL 1:10 TMUV strain Thai-MLO305 viral suspension containing 0.9 $\log_{10}$ plaque-forming units (PFUs) per mosquito (4.4 $\log_{10}$ PFUs/mL) and incubated at 27°C with apple slices provided as a carbohydrate source. Five mosquitoes were collected and homogenized individually at 0, 12, 24, 36, 48, and 60 hours as well as 3, 4, 5, 7, 11, 14, 21, and 28 days after inoculation. Mosquito samples were triturated in 2 mL diluent, split into two 1-mL aliquots, and then stored at −70°C. The qRT-PCR assays using the TMUV-NS5 primers and probe were performed with the mosquito homogenates and used to determine the amount of virus present. Non-template control water and *Cx. tarsalis* RNA were included in all assays as negative controls (Ct values ≥ 36).

RESULTS

Specificity of the conventional TMUV primer pair. To determine the specificity of the TMUVE1 forward and TMUVE1 reverse primer set, we tested the primers against a variety of flaviviruses, including four DENV (serotypes 1, 2, 3, and 4), WNV, and JEV as well as several viruses isolated from field-collected mosquitoes (a Kamiti River-like virus and another insect flavivirus-like virus) and two additional strains of TMUV. A single band was visualized for TMUV strains Thai-JSL385 and Thai1986, whereas no PCR product was visualized for samples from any of the other flaviviruses (Figure 2). These results indicate that TMUVE1 forward and TMUVE1 reverse primers specifically detected TMUV but did not recognize related flaviviruses.

Sensitivity and specificity testing of qRT-PCR assay. As indicated by the standard curve (Figure 3), there was a linear relationship between the Ct values determined by the qRT-PCR assay and serial 10-fold dilutions of TMUV RNA. In the evaluation of various dilutions of stock TMUV Table 2, the qRT-PCR was highly sensitive and able to detect < 1 PFU equivalent of TMUV. To test the specificity of the designed TMUV-NS5 qRT-PCR primer and probe, combinations of different templates, including two Thailand TMUV isolates and other known flaviviral RNAs, were tested using the qRT-PCR assay. Although the primers and probe readily detected the four strains of TMUV, including dilutions of up to 1:1,000,000 (< 1 PFU equivalent/mL), the TMUV-NS5 qRT-PCR primers and probe did not detect any of a variety of other flaviviruses, alphaviruses, or bunyaviruses (Table 3).

A qRT-PCR

Position	9080 9099	9112 9126	9139 9161
TMUV-NS5F / NS5P / NS5R	GAGGCGTTGAAGGACTTGGA	GGGTATGTGCTGCGT	AAAGAAGGAGGACTCATGTACGC
West Nile (NY99) DQ211652	GAGGTGTCGAGGGCTTGGGC	GGGTACATCCTGCGT	CGGCCTGGGGGCAAGATCTAT
Ilheus NC_009028	GTGGAGTCGAAGGGCAAGGA	GGGTACATTCTGAGG	CTGGAGGGTGGAAACATGTTTGC
Ntaya JX236040	GAGGAGTCGAGGGCTTAGGC	GGATACGTGCTGAGA	AATCCAGGAGGCCTCATGTATGC
Bagaza (DakAr B209)	GAGGCGTTGAAGGCATGGGC	GGGTATGTATTGAGG	AAGGAAGGGGGGCTCATGTAC
Duck Tembusu (ZJ 497) JQ314464	GAGGTGTTGAAGGACTTGGA	GGGTATGTGCTGCGT	AAAGAAGGAGGACTTATGTACGC
Tembusu (BDY-1) JF312912	GAGGTGTTGAAGGACTTGGA	GGGTATGTGCTGCGT	AAAGAAGGAGGACTTATGTACGC
Tembusu (MM1775) JX477685	GAGGTGTTGAAGGACTTGGA	GGATATGTGCTGCGT	AAGGAAGGGGGACTTATGTATGC
Tembusu (THCAr) AF013409	GAGGCGTTGAAGGACTTGGA	GGGTATGTGCTGCGT	AAAGAAGGAGGACTCATGTATGC
Tembusu (Thai08-093)	GAGGCGTTGAAGGACTTGGA	GGGTATGTGCTGCGT	AAAGAAGGAGGACTTATGTACGC
Tembusu (Thai08-381)	GAGGCGTTGAAGGACTTGGA	GGGTATGTGCTGCGT	AAAGAAGGAGGACTCATGTACGC
Tembusu (Thai08-454)	GAGGCGTTGAAGGACTTGGA	GGGTATGTGCTGCGT	AAAGAAGGAGGACTTATGTACGC
Tembusu (Thai-MLO305)	GAGGCGTTGAAGGACTTGGA	GGGTATGTGCTGCGT	AAAGAAGGAGGACTCATGTACGC
Tembusu (Thai-JSL385)	GAGGCGTTGAAGGACTTGGA	GGGTATGTGCTGCGT	AAAGAAGGAGGACTCATGTACGC

B Conventional PCR

Position	1556 1575	1971 1994
TMUVE1-FWD / REV	CGTGGCAAAACAGAGAGTCA	GATTATGGTAGAGGTGGAACCTCC
West Nile (NY99) DQ211652	TGTGGAGGAACAGAGAGACG	GGTCCTGATTGAATTGGAACCACC
Ilheus NC_009028	CGTGGAAGAACCGTGAGAGC	GATTCTGGTTGAACTTGAGCCCCC
Ntaya JX236040	ATTGGCAAAATAGAGAGTCA	AGTGATAGTTGAATTGGAACCACC
Bagaza (DakAr B209)	CATGGCAGAACAGAGAATCA	AGTGGTTGTTGAGCTTGAACCCCC
Duck Tembusu (ZJ 497) JQ314464	CATGGCAAAACAAAGAGTCA	GATAATGGTGGAAGTGGAACCTCC
Tembusu (BDY-1) JF312912	CATGGCAAAACAAAGAGTCA	GATAATGGTGGAAGTGGAACCTCC
Tembusu (MM1775) JX477685	CATGGCAAAACAGAGAGTCA	GATCATGGTAGAAGTGGAACCTCC
Tembusu (Thai08-093)	CATGGCAAAACAAAGAGTCA	GATAATGGTGGAAGTGGAACCTCC
Tembusu (Thai08-381)	CGTGGCAAAACAGAGAGTCA	GGTTATGGTAGAGGTGGAACCTCC
Tembusu (Thai08-454)	CATGGCAAAACAAAGAGTCA	GATAATGGTGGAAGTGGAACCTCC
Tembusu (Thai-MLO305)	CGTGGCAAAATAGAGAGTCA	GGTTATGGTAGAGGTGGAACCTCC
Tembusu (Thai-JSL385)	CGTGGCAAAACAGAGAGTCA	GATTATGGTAGAGGTGGAACCTCC

FIGURE 1. Alignment of sequences comparing the primers and probe binding locations for (**A**) qRT-PCR and (**B**) conventional PCR. Field isolates were aligned to GenBank sequences to determine percent identities. The primer and probe nucleotide positions are based on TMUV strain MM1775 (JX477685).

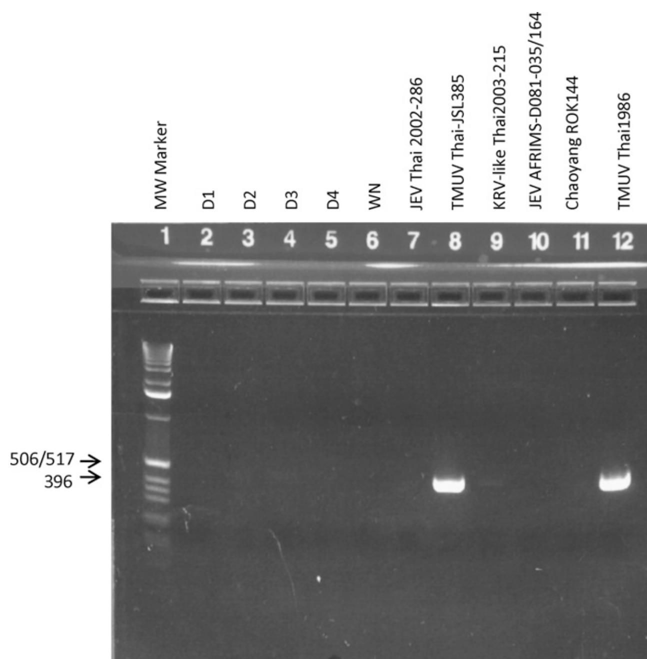

FIGURE 2. Conventional PCR and specificity testing of TMUV TMUVE1 forward and TMUVE1 reverse primers against dengue-1 (D1; lane 2), dengue-2 (D2; lane 3), dengue-3 (D3; lane 4), dengue-4 (D4; lane 5), West Nile virus (WN; lane 6), JEV Thai2003-286 (lane 7), TMUV Thai-JSL385 (lane 8), Kamiti River-like Thai2003-215 (lane 9), JEV AFRIMS-D081-035/164 (lane 10), Chaoyang ROK144 (lane 11), and TMUV Thai1986 (lane 12). Agarose gel electrophoresis of the PCR products and DNA molecular weight marker (lane 1) was performed using 2% E-gel gels. Expected TMUVE1 forward and TMUVE1 reverse primer PCR product is 439 bps.

TMUV strains Thai-MLO305 and Thai-JSL385 contained identical sequences in the NS5 primer and probe-binding regions and were used interchangeably during testing. This finding showed that TMUV-NS5 primers and probe could specifically detect TMUV, while not cross-reacting with other closely related flaviviruses.

To examine intra-assay variability, we tested 37 specimens in duplicate. For each of these pairs, the Ct values were similar and differed by a mean of 0.14 (median = 0. 09) and an SD of 0.20 Ct units. To examine interassay variability, we tested the same specimen in duplicate on 5 separate days. Again, the assay was consistent with a mean Ct reading of 24.6, an SD of 0.12, and a total range of 24.44–24.75 Ct values for 10 specimens tested on 5 separate days. This finding indicates that the assay was consistent, with little intra- or interassay variability.

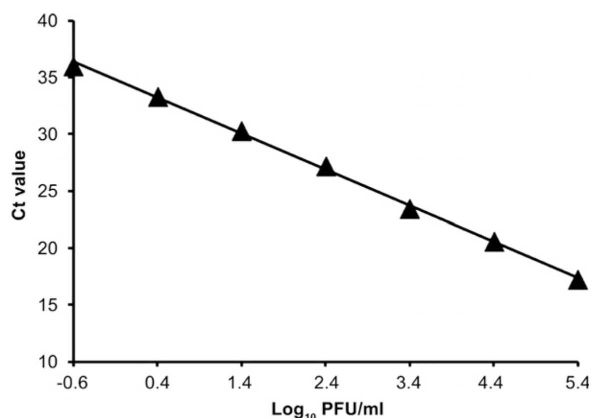

FIGURE 3. TMUV standard curve. Viral titer was determined by a standard plaque assay using Vero cells. The average Ct value (tested in duplicate) of 10-fold serial dilutions of TMUV RNA as determined by the NS5 qRT-PCR assay using the Thai-JSL385 strain of TMUV was plotted against the number of PFUs of virus present in the sample.

TABLE 2
Sensitivity of RNA viral detection using qRT-PCR and TMUV-NS5 primers and probe for selected strains of TMUV

Strain	PFU/mL	Dilution			
		Undiluted	10^{-2}	10^{-4}	10^{-6}
Thai-MLO305	5.3	13.0*	19.5	26.3	32.8
Thai-MLO305 cDNA	Undiluted†	13.7	21.6	28.4	NT
AFRIMS-D099-014/161	Undiluted	14.0	21.2	NT	NT

NT, not tested.
*Mean Ct value of duplicate tests of each sample.
†These viruses did not plaque in cell culture but were tested as undiluted cell culture suspensions unless otherwise indicated.

Replication of TMUV in mosquitoes. We used the TMUV-NS5 primers and probe in a qRT-PCR assay to test each of the five *Cx. tarsalis* mosquitoes collected at each time point. These data were used to generate a viral replication curve for TMUV in *Cx. tarsalis* (Figure 4). Because Ct values are inversely related to the amount of RNA present, we plotted the mean observed Ct value subtracted from 36 (value for a negative specimen) as a measure of the amount of viral RNA present. After an initial eclipse phase, RNA titers rapidly rose, peaking at about 4 days after inoculation, and remained high until the conclusion of the experiment on day 28 after inoculation (Figure 4). Because the viral titers shown in Figure 3 were based on virus grown in Vero cells and the growth curve is for virus grown in a mosquito, the number of genomic copies per PFU is only an estimate.

DISCUSSION

We developed conventional and qRT-PCR assays for detecting TMUV in cell culture supernatant and laboratory-infected mosquitoes that were both specific and sensitive. Although TMUV was first isolated in 1955 in Malaysia[3] and more recently, in regions of southeast Asia, including Thailand and China,[5–13] relatively little is known about this virus. We developed conventional PCR primers specific for the TMUV envelope gene and tested the primer pair to determine the most ideal conditions for detection of TMUV RNA. Our results showed that the TMUVE1 forward and reverse primer set detected TMUV RNA in a virus-specific manner, while not detecting a variety of other flaviviruses.

The TMUV-NS5 primer and probe set was highly sensitive, in that it was able to detect TMUV RNA from several different isolates, including from dilutions containing < 1 PFU equivalents. It was also highly specific in that it did not react when tested with a variety of other flaviviruses, including four DENVs, WNV, JEV, YFV, and two insect flaviviruses. These results indicated the TMUV-NS5 primer and probe set is ideal for use as a rapid and specific assay for the detection of TMUV in both the laboratory and field settings.

TMUV has been isolated from a variety of *Culex* spp.,[11,22] and *Cx. vishnui* are able to transmit this virus in the laboratory.[13] The qRT-PCR developed here readily indicated that, after intrathoracic inoculation, there was a definite eclipse

Table 3
Detection of viral RNA using qRT-PCR and TMUV-NS5 primers and probe

Virus	Strain	Titer PFU/mL*	Ct value
Tembusu	Thai-MLO305	5.3	13.0
Tembusu	AFRIMS-D099-014/161	Undiluted†	14.0
Tembusu	Thai-93	3.6	26.7
Tembusu	Thai-385	Not tested	20.9
Tembusu	Thai-MLO305 cDNA	Undiluted†	13.7
Other members of the genus *Flavivirus*			
Dengue-1	HAW	7.6	> 36
Dengue-2	S16803	8.9	> 36
Dengue-3	H87	5.1	> 36
Dengue-4	H-421	5.6	> 36
Insect flavivirus‡	IF568	Undiluted†	> 36
Japanese encephalitis	ROK-2.0221	5.6	> 36
Kamiti River-like‡	Thai2003-215	Undiluted†	> 36
St. Louis encephalitis	Fort Washington	5.6	> 36
West Nile	NY397-99	8.9	> 36
Yellow fever	Asibe	3.6	> 36
Members of the genus *Alphavirus*			
Chikungunya	Indo 23574	6.6	> 36
Getah	ROK-2.0017	6.2	> 36
Mucambo	PE-4.0904	6.0	> 36
O'nyong'nyong	Gulu	6.1	> 36
Ross River	R-495	6.4	> 36
Sindbis	AR 339	5.6	> 36
Trocara	PE-7.0009	3.8	> 36
Venezuelan equine encephalitis	TC-83	6.1	> 36
Members of the family *Bunyaviridae*			
Caraparu	PE-18.0572	3.9	> 36
Crimean Congo hemorrhagic fever	10200	5.0	> 36
Itaqui	PE-0.0036	3.9	> 36
La Crosse	97-WV-131	4.4	> 36
Murutucu	PE-1.0735	4.4	> 36
Rift Valley fever	ZH601	5.6	> 36

*Viral titers were determined in six-well plates of Vero cells.
†These viruses do not plaque in cell culture but were tested as undiluted cell culture suspensions unless otherwise indicated.
‡IF568 was isolated from *Cx. nigropunctatus* from Thailand in April of 2008, and Thai2003-215 was isolated from *Cx. gelidus* from Thailand in February of 2003.

phase followed by rapid replication of TMUV in *Cx. tarsalis*. These data showed that the TMUV-NS5 qRT-PCR assay is quantitative, and it is an ideal assay for observing viral replication in laboratory-infected and field-collected mosquitoes. Additionally, the TMUV-NS5 qRT-PCR assay was tested in a field setting in Thailand in 2011. Although there were no positive isolates identified, the assay did not produce any false-positive results for 15 *Culex* spp. mosquito pools that were tested. Because TMUV is an emerging flavivirus that is currently circulating in several southeast Asian countries and

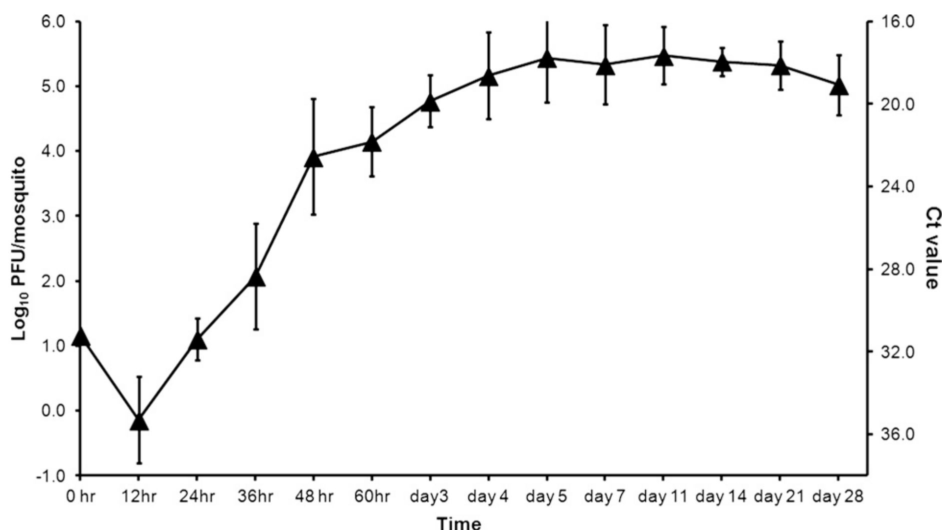

Figure 4. Replication of TMUV in *Cx. tarsalis* mosquitoes after intrathoracic inoculation. At each time point, five mosquitoes that had been inoculated with the Thai-MLO305 strain of TMUV were collected, homogenized individually, and tested using qRT-PCR for the TMUV-NS5 primers and probe. Viral titers were estimated based on the standard curve (Figure 3).

may pose a potential future disease risk, the rapid and specific detection of TMUV RNA in mosquitoes will enable health-care providers to improve methods for determining potential human health hazards, especially in field environment settings. We described the development of sensitive and specific conventional and qRT-PCR assays for detection of TMUV in field-collected mosquitoes that can be used to enhance surveillance efforts. Additionally, testing of these assays in regions with active TMUV circulation will better determine the effectiveness of these assays.

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