RESEARCH NOTE

ISOLATION OF TEMBUSU VIRUS FROM *CULEX QUINQUEFASCIATUS* IN KANCHANABURI PROVINCE, THAILAND

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Abstract. Tembusu virus (TMUV) is a positive-sense, single-stranded RNA virus belonging to Ntaya virus serogroup of Flaviviridae family. Of 1,478 female *Culex* mosquitoes collected from Mueang Kanchanaburi, Thailand using BG Sentinel Trap, 91.5% were *Cx. quinquefasciatus.* One pool from seventy pools of mosquitoes was TMUV-positive by reverse transcriptase-PCR and virus isolation. This is the first report of an isolation of TMUV from *Cx. quinquefasciatus* collected near a chicken farm, providing evidence for TMUV transmission by this *Culex* species in a natural habitat. This has implications for possible infection in humans.

Keywords: *Culex quinquefasciatus*, chicken farm, Tembusu virus, Thailand

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INTRODUCTION

Tembusu virus (TMUV) is a positive-sense single-stranded RNA virus belonging to the Ntaya virus serogroup of Flaviviridae family (Thontiravong *et al*, 2015). It was first isolated in Malaysia in 1955 from *Culex tritaeniorynchus* (O'Guinn *et al*, 2013). TMUV was also isolated from *Cx. vishnui* and *Cx. vishnui* subgroup mosquitoes in Malaysia in 1970 (Platt *et al*, 1975) and from population pools of *Cx.*

vishnui, Cx. tritaeniorynchus, and *Cx. gelidu*s (O'Guinn *et al*, 2013). In 1992, TMUV was isolated from *Cx. tritaeniorynchus* collected in Chiang Mai, Thailand (Pandey *et al*, 1999). In 2002, the Armed Forces Research Institute of Medical Sciences (AFRIMS) collected from Kong Mong Tha-Sangkhla Buri, Kanchanaburi Province, Thailand two pools of *Cx. vishnui* positive for TMUV (O'Guinn *et al*, 2013).

TMUV has had a significant impact on duck industry in China (Yan *et al*, 2011) and in Thailand (Thontiravong *et al*, 2015; Chakritbudsbong *et al*, 2015) identified a new duck Tembusu virus (DTMUV). Clinical signs in ducks include such neurological manifestations as inability to stand, ataxia and paralysis (Thontiravong *et al*, 2015). A chicken TMUV isolate, originally named Sitiawan virus, can also cause encephalitis and retarded growth in broiler chickens (Kono *et al*, 2000).

Cx. quinquefasciatus is predominantly an urban mosquito but also is the most common domestic mosquito species of semi-urban and rural areas (Mariappan *et al*, 2014). It is extremely abundant and geographically in tropical countries (Nitatpattana *et al*, 2005). Here, we report the isolation of TMUV from *Cx. quinquefasciatus* in Thailand. This has important zoonotic implication for humans.

MATERIALS AND METHODS

Field site location

Mosquitoes were collected from rice paddy field and a small chicken farm near the Veterinary and Agriculture Division, Veterinary and Remount Department, Royal Thai Army, Ministry of Defense, Ko Samrong Sub-district, Mueang, Kanchanaburi Province (13° 58′ 18.6″ N, 99° 30′ 40.4″ E), Thailand**,** located 300-400 m from Khwae Noi River.

Mosquito collection

Adult mosquito collections were made two nights weekly from 5 August to 7 October, 2015 employing a BG-Sentinel trap (BioGents, Regensburg, Germany) (Roiz *et al*, 2012). This device mimics convection currents created by a human body, employing an attraction visual cue and release of odors about 20 meters (Pombi *et al*, 2014).

Virus isolation and identification

Mosquitoes were transported to the laboratory in liquid nitrogen. Female mosquitoes were divided into groups of specific species of 20-30 individuals (O'Guinn *et al*, 2004). A group of mosquitoes was ground and homogenized in 1 ml aliquot of sterile phosphate-buffered saline pH 7.4 (PBS) and 30% fetal calf serum (HyClone, South Logan, UT). Homogenate was centrifuged at 1,300*g* for 15 minutes at 4ºC using a filter tube (Spin-x, 0.22 micron Costar 8160, Tewksbury, MA) and stored at -70ºC until used. A 0.2 ml thawed aliquot was inoculated onto a suspension of 3-day old C6/36 cell line (ATCC® CRL-1660™) and incubated at 32ºC for 90 minutes under an atmosphere of 5% CO_2. Then 4 ml aliquot of MEM media (GIBCO, Waltham, MA) was added and cell suspension incubated as described above for 7 days (Nitatpattana *et al*, 2005).

Virus-infected C6/36 cells were deposited onto Teflon coated slides, which were air dried inside a bio-safety cabinet and fixed in chilled acetone for 10 minutes at -20°C. Slides were overlaid with 30 µl of monoclonal anti-flavivirus (4G2) antibodies (ATCC, Manassas, VA) and incubated in a moist chamber at 37°C for 30 minutes before being washed three times with PBS. The bound antibodies were detected with fluorescein-isothiocyanate (FITC)-conjugated goat anti-mouse IgG

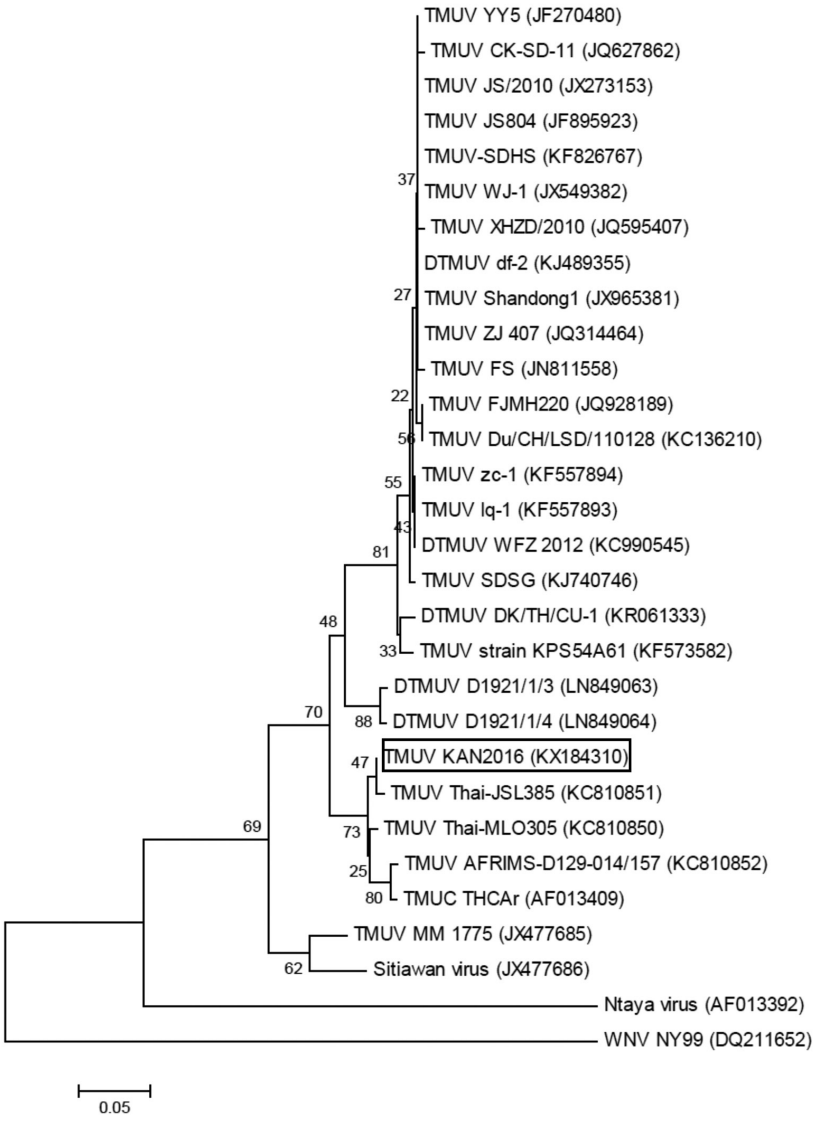

Fig 1–Neighbor-joining phylogenetic tree based on NS5 gene (239 bp) of TMUV KAN2016 and those of other TMUV isolates deposited in GenBank. Ntaya virus (AF013392) and WNV NY99 (DQ211652) were used as outliners. The phylogenetic tree was generated using maximum composite likelihood method with 1,000 bootstrap replicates. Number at branch junction indicates percent bootstrap replicate. Scale bar indicates percent sequence change (0.05 = 5%).

(Sigma-Aldrich, St Louis, MO) diluted 1:40. The slides were washed, mounted and examined under a fluorescence microscope (Henchal *et al,* 1983).

RNA was extracted from cells from a positive culture using QIAamp Viral RNA Mini Kit (QIAGEN, Germantown, MD). CDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA) following manufacturer's instructions. Flavivirus-specific primers PF1S (5′-TGYRTBTAYAACATGATGGG-3′ and PF2R (5′-GTGTC CCADCCDGCDGTRTC-3′) (where B = C, G, T; D = A,G,T; R = A,G; and Y = C,T) (Moureau *et al,* 2007) were used to detect flavivirus in samples. In brief, a 50-µl reaction mixture containing 5 µl of viral cDNA, 0.5 µl of each primer (10 µM) and 44 µl of reaction mixture (KAPA biosystems, Wilmington, MA) was subjected to 35 cycles of 95ºC for 30 seconds, 55ºC for 30 seconds and 72ºC for 30 seconds (Tpersonel 48 thermalcycler, Biometra, Göttingen, Germany). The 239-bp Flavivirus amplicon was purified by 2% agarose gel-electrophoresis and visualized with SYBR Green dye (Thermo Fisher Scientific). Gel-purified amplicons

were directly sequenced (First BASE Laboratories, Seri Kembangan, Selangor, Malaysia), and the sequences were submitted to GenBank. Nucleotide sequences were compared with those from GenBank using BLAST analysis (www.ncbi.nlm.nih.gov) and phylogenetic tree was constructed with MEGA 6.0 software (1,000 bootstraps) (Tamura *et al*, 2013).

RESULTS

Between 5 August to 7 October 2015, 2,201 *Culex* mosquitoes (1,478 females) were collected by BG-Sentinel Traps in Mueang Kanchanaburi. Female *Cx. quinquefasciatus* and *Cx. sitiens* constituted 1,353 (91.5%) and 125 (8.5%), respectively.

TMUV was isolated only from *Cx. quinquefasciatus* in 1/70 (1%) pool by IDFA and RT-PCR (data not shown). DNA sequencing and BLAST analysis of TMUV KAN2016 (GenBank accession no. KX184310) in C6/36 cells showed highest nucleotide identity of 98% and 99% with Thai-MLO305 (KC810850) and Thai JSL-385 (KC810851), respectively. Phylogenetic analysis revealed TMUV KAN2016 is closely related to recent isolates of TMUV in the same province (Fig 1).

DISCUSSION

Cx. quinquefasciatus is endemic in all tropical areas and is known to be highly anthropophilic (Charlwood, 1979) with an ability to adapt to the anthropic environment (Almirón and Brewer, 1996). *Cx. quinquefasciatus* was extracted from chicken feces (Cooperband *et al*, 2008). Currently, Thailand has a number of industrial chicken farms spread all over the country.

This is the first report of TMUV virus isolated from *Cx. quinquefasciatus* collected near the small chicken farm in Thailand. From phylogenetic analysis TMUV KAN2016 is closely related to leghorn chicken TMUV MLO305 and JSL-385, which cause growth retardation and death in young chickens (O'Guinn *et al*, 2013). This result supports the notion that TMUV can be transmitted by *Cx. quinquefasciatu*s to chickens in Thailand. However, TMUV KAN2016 is not in the same DTMUV group isolated from ducks in China, Malaysia and Thailand (Yan *et al*, 2011; Homonnay *et al*, 2014; Chakritbudsbong *et al*, 2015; Thontiravong *et al*, 2015). TMUV isolates were also detected from pools of *Cx. gelidus*, *Cx. tritaeniorynchus* and *Cx. vishnui* in Thailand (Leake *et al*, 1986; Pandey *et al*, 1999). DTMBV transmitted by mosquitoes poses a potential threat to the poultry industry in Thailand and neighboring countries and it will be necessary to implement preventive measures to avoid economic loss.

Although TMUV has not been reported to cause disease in humans, recently in China DTMUV infection has been reported in groups at high risk for duck-to-human transmission without signs of illness (Tang *et al*, 2013). Thus, there is an urgent need to conduct surveillance of TMUV in high risk human populations in Thailand.

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