Short Communication

Detection of West Nile Virus in Mosquitoes in Nakhon-pathom and Phetchaburi Province, Thailand

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Abstract

West Nile (WN) disease is one of the encephalitic diseases in humans and mammals. This disease has been reported in several areas of the world. Thailand is located in the area of high risk of WN virus (WNv) infection concerning a natural transmission cycle. However, the WNv infection has not been previously reported. Therefore, the aim of this study was to detect WNv in mosquitoes in some areas in Nakhon-pathom and Phetchaburi provinces, central Thailand. The period of this study was done during November 2008 to November 2010. We found that there were four genera collected including Aedes, Anopheles, Culex and Mansonia. *Culex spp* such as *Culex gelidus* and *Culex quinquefasciatus* was abundant and frequently collected. Fifty mosquitoes collected from each of the fifty pools were tested for WNv by using the antigen test kit (Vectest®) and RT-PCR. All of the samples were negative for WNv in both methods. This is a preliminary report regarding WNv in mosquitoes in Thailand and concerned for epidemiology. Further study should be done to continue a close surveillance for the presence of the WNv in the natural transmission host.

Keywords: detection, mosquitoes, RT-PCR, VecTest®, West Nile virus

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บทคัดย่อ

การตรวจหาเชื้อไวรัสเวสท์ไนล์ในยุง ในพื้นที่จังหวัดนครปฐมและจังหวัดเพชรบุรีของประเทศไทย

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โรคเวสท์ไนล์ จัดเป็นโรคที่ก่อให้เกิดภาวะสมองอักเสบในมนุษย์ และสัตว์เลี้ยงลูกด้วยนม พบรายงานอย่างเป็นทางการของการระบาดโรคเวสท์ไนล์ในพื้นที่ต่าง ๆ ทั่วโลก แต่ไม่พบรายงานของการพบเชื้อไวรัสเวสท์ไนล์ในประเทศไทย แม้ว่าประเทศไทยจะตั้งอยู่ในพื้นที่เสี่ยงของวัฏจักรการเกิดโรคเวสท์ไนล์ ดังนั้นวัตถุประสงค์ของการศึกษาครั้งนี้คือ เพื่อสำรวจหาเชื้อไวรัสเวสท์ไนล์ใน ตัวอย่างยุงในแถบภาคกลางของประเทศ ภายในพื้นที่จังหวัดเพชรบุรี และจังหวัดนครปฐม ในระหว่างเดือนพฤศจิกายน พ.ศ. 2551 ถึง เดือนพฤศจิกายน พ.ศ. 2553 ชนิดของยุงที่ถูกดักจับได้ประกอบด้วย *Aedes*, *Anopheles*, Culex และ Mansonia โดยเฉพาะสายพันธุ์ Culex เช่น Culex *gelidus* และ *Culex quinquefasciatus* จัดเป็นกลุ่มตัวอย่างที่ถูกดักจับมากที่สุด ตัวอย่างยุงกลุ่มละ 50 ตัวอย่าง จำนวนทั้งหมด 50 กลุ่มตัวอย่างมาตรวจหาเชื้อไวรัสเวสท์ไนล์โดยอาศัยชุดตรวจสอบยี่ห้อ Vectest® กรณีที่ตรวจพบเชื้อไวรัสเวสท์ไนล์ด้วยชุดตรวจสอบข้างต้น จะทำการยืนยันผลซ้ำด้วยวิธีอาร์ที พีซีอาร์ ผลการศึกษาครั้งนี้ไม่พบเชื้อไวรัสเวสท์ไนล์ในทุกกลุ่มตัวอย่างของยุงที่ทำการตรวจสอบจากทั้งชุดตรวจสอบยี่ห้อ Vectest® และวิธีอาร์ที พีซีอาร์ การศึกษาครั้งนี้จัดว่าเป็นการศึกษาเบื้องต้นเกี่ยวกับเชื้อไวรัสเวสท์ไนล์ในประเทศไทย ข้อมูลที่ได้ในการศึกษาครั้งนี้จัดเป็นข้อมูลด้านระบาดวิทยา สำหรับการเฝ้าระวังอุบัติการณ์ของโรคเวสท์ไนล์ในโฮสต์ที่เป็นกลุ่มเสี่ยงตามธรรมชาติต่อไป

คำสำคัญ: การตรวจหา เชื้อไวรัสเวสท์ไนล์ ยุง Vectest® และ อาร์ที พีซีอาร์

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Introduction

The West Nile virus (WNv) is a mosquito transmitted Flavivirus and closely related to other mosquito-transmitted human pathogens such as Japanese encephalitis virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, and Kunjin viruses (Burke and Monath, 2002) The WNv can cause fever and in some cases critical encephalitis or meningitis in humans and mammals (Reisen et al., 2004). WNv has been reported to be endemic in many areas such as America (Nasci, 2001), Europe (Kolman, 1973), Africa (Fagbami 1978; Miller et al., 2000), Russia (Platonov, 2001), Asia and Australia (Kanamitsu et al., 1979; Petersen and Roehrig, 2001) where mosquitoes are active.

The WNv has been maintained in a natural transmission cycle involving a variety of bird and mosquito species (Marra et al., 2004; Turell et al., 2005). A rapid antigen-capture wicking assay in a dip stick format (VecTest ®, Medical Analysis Systems, Inc., Camarillo, CA, USA) was introduced for detecting WNv in mosquito pools (Nasci et al., 2002; Ryan et al., 2003). PCR-based molecular has been used for detecting WNv in mosquitoes (Lanciotti et al., 2000; Anwar et al., 2006; Lampman et al., 2006; Rondini et al., 2008).

Culex mosquitoes play a major role in the transmission cycle of WNv although various species of the Aedes genus are also capable of causing the infection (Yvette et al., 2010). The virus has been detected in several Asian countries (Igarashi 1987; Paramasivan et al., 2003). In Thailand, the WNv infection has not been officially reported. However, The WNv should be surveyed in the area of high risk of WNv infection concerning a natural transmission cycle. Therefore, the aim of this study was to detect WNv in Thailand by using the antigen test kit (VecTest®) and reverse transcriptase-polymerase chain reaction (RT-PCR) in the homogenized mosquito's pool samples collected from Nakhon-pathom province and Petchburi province, Thailand where it is possibly a risky area of the virus distribution.

Materials and Methods

Mosquito collection: Mosquitoes were captured within two areas including Kasetsart University, Nakhon-pathom province (global positioning system: 47P 0606462 UTM 1549372) and the Laem Phak Bia Environmental Study and Development Project, Phetchaburi province (global positioning system: 47P 0618398 UTM 1442785) (Fig 1). Mosquito sampling was carried out from November 2008 to November 2010 using mosquitoes light trap (model Akio-2000, Japan) which operated from 5 p.m. to 7 a.m. on each day of field study. Each mosquito sample was identified according to Rattanarithikul and Panthusiri's study (1994). Each mosquito pool was homogenized and processed for WNv detection by an immunochromatography test kit, (Vectest®, Medical analysis systems, Inc., Camarillo, CA, USA) and RT-PCR.

Figure 1 Mosquitoes were captured within two areas including Kasetsart university, Nakhon-pathom province (■) and the Laem Phak Bia Environmental Study and Development Project, Phetchaburi province (●).

West Nile Virus Antigen Assay: The WNv Antigen Assay is based on the dual monoclonal antibody "sandwich" technique. The test is initiated by placing one VecTest® WNv dipstick into 250 microliter (µl) of ground mosquito extract. Antigen that is present in the solution is bound to the specific antibody with a gold sol particle label. As the antigen-antibody-gold complexes migrate through the test zone containing immobilized WNv antibody, they bind to the immobilized antibody forming a "sandwich". The unbound dye complexes migrate out of the test zone and can be captured later in the control zone. A reddish-purple line develops on the specific area of the test zone when antigen is present. The control line, farthest from the sample, should always develop provided the test has been carried out correctly (Nasci et al., 2002; Ryan et al., 2003). The VecTest® was evaluated for sensitivity and specificity in mosquito sample as 65 percentages and 98 percentages, respectively (Burkhalter et al., 2006).

The samples were processed according to protocol of the test. Briefly, 50 mosquitoes were placed in a plastic culture tube and then 2,500 µl of grinding solution were dispensed onto the mosquitoes with four copper-coated BBs provided in the kit. The capped tube was vortex for 1 min at high speed until the mosquito pool was homogenized into slurry (a centrifugation step may be performed to remove excess mosquito debris before running the test). 250 µl of mosquito homogenate was placed into a conical tube provided, then place a test strip from the canister with the arrows pointing down (replace the desiccant cap on the canister to protect the remaining strips from moisture). The completed reaction should be left for 15 min at room temperature. The presence of a control line alone on the dipstick indicates a negative test result. The presence of two lines indicates a positive result of WNv antigen detection. The result should be observed within 30 minutes after applying the strip into the conical tube.

Viral ribonucleic acid extraction: Viral ribonucleic acid (RNA) was extracted by using PureLink TM Viral RNA/DNA Kits (Invitrogen, USA) according to its manufacturer's recommendation. Briefly, 200 µl of homogenized mosquitoes were broken by 25 µl of proteinase K and 200 µl of lysis Buffer (containing 5.6 µg, Carrier RNA) into a sterile microcentrifuge tube at 56°C for 15 min. For binding and washing step, the lysated mosquitoe homogenized sample was prepared by using 250 µl of 96-100% ethanol and wash buffer onto the Viral Spin Column. Then, the Viral Spin Column was centrifuge at ~6,800 x g for 1 min and discarded the washing tube containing the flow-through was discarded. Finally, according to elution step, the viral spin column was placed into the recovery tube and 30 µl of sterile, RNase-free water was added to the center of the column. The column was incubated at room temperature for 1 min and then was centrifuged at maximum speed for 1 min. Now, purified viral nucleic acids were solved into the recovery tube. Purified viral nucleic acid solution was stored at -80°C until tested by RT-PCR.

West Nile detection by reverse transcriptase polymerase chain reaction: RNA samples extracted from 50 mosquitoes from each the 50 pools, were detected by using conventional RT-PCR. A pair of specific primer for E gene of WNv was forward primer: 5'-CGG CGC CTT CAT ACA CAC–3' and reverse primer: 5'–GCC TTT GAA CAG ACG CCA T–3' (modified from Lanciotti et al., 2000). RT-PCR cycling conditions were 45 min at 45°C for the reverse transcription step and 5 min at 92°C for the initial PCR activation step followed by 35 cycles of 1 min at 92°C (denaturation), 1 min at 55°C (annealing), 1 minute at 72°C (extension), and 5 min at 72°C for the final extension. RT-PCR mixtures were performed in 25 µl reaction. One and a half microliters of RNA were mixed with 12.5 µl of 2x reaction mix (a buffer containing 0.4 millimolar (mM) of each dNTP, 3.2 mM MgSO4; Invitrogen, Carlsbad, CA), 0.5 µl of each

forward and reverse primer, 10.5 µl of purified viral nucleic acids sample and 1 µl of SuperScript III Reverse transcriptase/ Platinum Taq Mix (Invitrogen Carlsbad, CA) RNA was amplified by using thermocycler (Perkin Elmer Cetus 9600, Perkin Elmer, Waltham, MA). PCR products included mosquitoes pooled sample, distillate sterile water as negative control, and the approximately 440-base pairs of Human WNv strain NYS 2001-6263, catalog number: NATWNV-0005 (50,000 copies/ml), NATrol™ (ZeptoMetrix Corporation, NY, USA) as positive control (Fig 2). PCR products were analyzed in 1.5% agarose gel (UltraPure™, Invitrogen, Carlsbad, CA) in 1x Tris base buffer at 100 volts for 25 min and were stained with 0.5 µg/ml of Ethidium Bromide and observed under ultraviolet light (UV Transilluminator 1000, Biorad, Inc.).

Results and Discussion

In this study, a total of four genera of mosquitoes sampled were identified including Aedes, Anopheles, Culex, and Mansonia. Culex spp. were trapped including *Culex gelidus* (42.29%), *Culex quinquefasciatus* (34.75%), and *Culex tritaeniorhynchus* (19.02%), while the least abundant species were *Aedes lineatopennis* (1.25%), *Aedes albopictus* (0.43%), *Anopheles barbirostris* (0.21%), *Mansonia uniformis* (0.14%) and other species (1.92%).

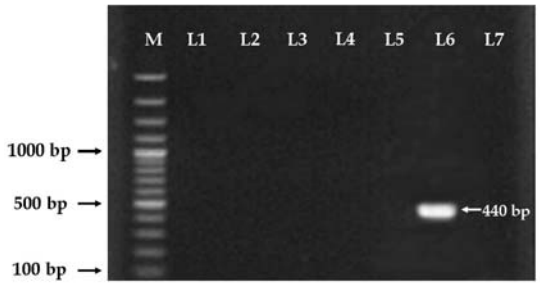

Figure 2 PCR products were analyzed in 1.5% agarose gel. M: 100 bp molecular weight marker; L1 to L5: each mosquitoes pooled samples; L6: positive control band approximately 440 bp and L7: distillate sterile water as negative control.

All mosquito pooled samples were negative for WNv by Vectest® or RT-PCR (Fig 2). Although this study was done continuously for 2 years, no positive sample was found. This indicated that WNv was not present in the areas throughout the study. However, to obtain information of infection or circulation of the virus, serological study might be helpful to gain the infection data of the virus. WNv has been reported in several countries in Asia where mosquitoes are plentiful and active (Igarashi 1987; Paramasivan et al., 2003). Our study showed that the presence of WNv was not directly correlated with the density of birds or mosquitoes in the area studied. We hypothesized that the area studied might be endemic for flavivirus since the area studied, especially Laem Phak Bia Environmental Study and Development Project, Petchburi province had a plentiful variety of wild birds and mosquitoes. The WNv circulation in any area depends on infected animals, vectors and susceptibility of host (Petersen and Roehrig, 2001).

Although this study was performed in a confined area, the survey had been continuously done for 2 years. The negative result indicated absence of Flavivirus circulation in the area studied. Other studies have indicated that the minimum field infection rate assumes that a single mosquito within a pool is infected (Gu et al., 2003). However, a more accurate estimate of infection rate was achieved using the maximum likelihood estimates for the probable infection rate that is based on the binomial distribution (Gu et al., 2004). Although there was no positive for WNv detection in this study, the investigation or surveillance of the virus still needs to be done.

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