รายงานวิจัยฉบับสมบูรณ์

โครงการ "GENETIC DIVERSITY AND ORAL SUSCEPTIBILITY TO DENGUE TYPE 2 OF *AEDES AEGYPTI* FROM HIGH AND LOW DENGUE RISK AREAS"

โดย DR. RONALD ENRIQUE MORALES VARGAS และ ดร. นพมณี เชื้อวัชรินทร์

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สัญญาเลขที่ BRT R_245004

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สนับสนุนโดยโครงการพัฒนาองค์ความรู้
และศึกษานโยบายการจัดการทรัพยากรชีวภาพ
ในประเทศไทย (โครงการ BRT)

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โครงการนี้ได้รับการสนับสนุนจากทุนวิจัยโครงการพัฒนาองค์ความรู้และศึกษานโยบายการจัดการทรัพยากรชีวภาพในประเทศไทย (โครงการ BRT) และ หน่วยวิจัยพาหะและโรคที่นำโดยพาหะ ศูนย์วิจัยเพื่อความเป็นเลิศ คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล, รศ. ศิริลักษณ์สุวรรณวงศ์ คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล พร้อมกันนี้ข้าพเจ้าขอขอบคุณ ดร. สุธี ยกส้าน ผู้อำนวยการศูนย์วิจัยและพัฒนาวัคซีน มหาวิทยาลัยมหิดล ศาลายา ที่ให้สถานที่ในการเตรียมและแยกไวรัสไข้เลือดออก, คุณสุรภี อนันตบรีชา ในหน่วยงาน ARBOVIRUS ของศูนย์วิทยาศาสตร์การแพทย์ จังหวัดนนทบุรี, คุณพัฒนาวรรณ ลิ้มสกุลศิริรัตน์ ที่ศูนย์วิทยาศาสตร์การแพทย์ จังหวัดชลบุรี สำหรับตัวอย่างไวรัสไข้เลือดออกและคณะผู้ร่วมงานทุกท่านที่จังหวัดฉะเชิงเทราและคณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล

ผู้จัดทำ

31 สิงหาคม 2546

บทคัดย่อและบทสรุป (Abstract and Summary)

รหัสโครงการ: BRT R_245004

ชื่อโครงการ: การศึกษาความหลากหลายทางพันธุกรรมและการยอมรับการติดเชื้อไวรัสไข้เลือดออกชนิด 2 โดยการกินในยุงลายชนิด *Aedes aegypti* ในบริเวณที่มีความเสี่ยงต่อการติดเชื้อสูงและต่ำ

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วัตถุประสงค์: เพื่อทำความเข้าใจปัจจัยที่มีผลต่อการติดเชื้อไข้เด็งกี่ (DEN) ในยุงพาหะ งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาความสามารถในการติดเชื้อทางการกินและ growth analysis ของเชื้อไข้เด็งกี่ชนิดที่ 2 สายพันธุ์เอเชียตะวันออกเฉียงใต้ ซึ่งแยกมาจากผู้ป่วยไข้เด็งกี่ที่มีความรุนแรงของโรคต่างๆกัน ในยุงลาย *Aedes aegypti* สายพันธุ์ที่ได้จากสภาพภูมิศาสตร์ต่างๆกัน กล่าวคือจากสถานที่ที่มีความเสี่ยงต่อการระบาดของโรคสูงและต่ำ นอกจากนี้ยังศึกษาโครงสร้าง และความหลากหลายทางพันธุกรรมของยุงลาย *Aedes aegypti* ซึ่งเก็บตัวอย่างจากสถานที่ที่มีความเสี่ยงสูงและต่ำต่อการระบาดของโรค เพื่อบ่งชี้ถึงการกระจายตัวของโครงสร้างทางพันธุกรรมนั้นๆในประชากรยุงลายต่อการแพร่ระบาดของโรค ความรู้จากงานวิจัยนี้จะนำไปสู่ความเข้าใจความสัมพันธ์ของเชื้อไวรัสเด็งกี่กับยุงลายพาหะ เพื่อที่จะนำไปสู่การพัฒนาหาวิธีการกำจัดเชื้อเด็งกี่และจำกัดพื้นที่เสี่ยงต่อการระบาดของโรคไข้เด็งกี่และไข้เลือดออกเด็งกี่ เนื่องมาจากความสามารถในการติดเชื้อของยุงลาย *Ae. aegypti* ในพื้นที่ที่ศึกษา

งานวิจัยนี้ได้ศึกษาเปรียบเทียบความสามารถต่อการติดเชื้อของยุง 8 สายพันธุ์จากพื้นที่ที่มีความเสี่ยงสูงและต่ำต่อการแพร่ระบาดของโรคไข้เด็งกี่ (DF) ไข้เลือดออกเด็งกี่ (DHF)/ไข้เด็งกี่ช็อค (DSS) ในจังหวัดฉะเชิงเทรา ภาคตะวันออกของประเทศไทย ยุงลายสายพันธุ์ต่างๆนั้นจะนำมาทดลองการติดเชื้อทางการกิน โดยใช้เชื้อเด็งกี่ชนิดที่ 2 จำนวน 4 สายพันธุ์ซึ่งแยกจากผู้ป่วยไข้เด็งกี่ที่มีความรุนแรงของโรคต่างๆกันกล่าวคือ ไข้เด็งกี่ธรรมดา (DF) และไข้เด็งกี่ช็อค (DSS) จากการศึกษาพบความแตกต่างอย่างมีนัยสำคัญต่อการติดเชื้อเด็งกี่ทั้ง 4 ชนิดในยุงสายพันธุ์ต่างๆกัน กล่าวคือเมื่อศึกษาอัตราการติดเชื้อทั้งหมดในยุงลายสายพันธุ์ต่างๆพบว่า ยุงลายสายพันธุ์จากเขตชนบทมีอัตราการติดเชื้อสูงกว่ายุงลายสายพันธุ์จากเขตเมืองอย่างมีนัยสำคัญ และเชื้อเด็งกี่ที่แยกจากผู้ป่วยไข้เด็งกี่ธรรมดา (DF) นั้นมีความสามารถในการติดเชื้อสูงกว่าเชื้อเด็งกี่ที่แยกจากผู้ป่วยไข้เด็งกี่ช็อค (DSS) การศึกษานี้ชี้ให้เห็นว่าการแพร่ระบาดของเชื้อเด็งกี่นั้นขึ้นอยู่กับทั้งสายพันธุ์ของเชื้อเด็งกี่และสายพันธุ์ของยุงลายพาหะ

Project Code: BRT R_245004

Project Title: Genetic diversity and oral susceptibility to dengue type 2 of *Aedes aegypti* from high and low dengue risk areas

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Project Period: 1 August 2002 – 31 July 2003

Objectives: In attempts to better define dengue (DEN) virus infection in its mosquito vector, this study is aimed: to clarify the dynamics of susceptibility and growth analysis of DEN-2 Southeast Asian genotype isolated from patient exhibiting different disease severity in orally infected *Aedes aegypti* originated from different geographic origin, indeed from high and low dengue risk areas. In addition, the genetic structure and the genetic variation it will be also studied, to identify the contribution of the genetic structure of *Ae. aegypt*i population to the pattern of dengue fever transmission. The basic knowledge of this study will, therefore, gives us the better understanding of dengue virus/*Ae. aegypti* relationship, in order to development the strategies for surveillance of dengue and delimitation of areas on risk for dengue/dengue hemorrhagic fever inferred from the susceptibility of *Ae. aegypti* from determined area.

The comparative susceptibility was carried out in 8 strains of *Ae. aegypti* from areas with high and low incidence of DF and DHF/DSS background in Chachoengsao province, East of Thailand. *Ae. aegypti* strains were challenged orally with four dengue type 2 isolate viruses recently isolated from patients exhibiting different disease severity, classical dengue fever (DF) and dengue shock syndrome (DSS). Significant variation in susceptibility to four isolates was observed among the *Ae. aegypti* strains tested. Overall infection rate analysis showed that the *Ae. aegypti* strains collected in urban areas seems like to be significantly higher susceptible than that *Ae. aegypti* from rural areas. Significant different infectivity between isolates virus were observed. The isolates viruses isolated from patients exhibiting classical dengue fever (DF) symptoms showed to be more infective than the isolates viruses isolated from patients exhibiting severe dengue shock syndrome (DSS) symptoms. These results may suggest that the efficacy of dengue virus circulation is likely to vary according to combination of the virus strains circulating and origin of the vector mosquitoes.

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1. บทนำ (BACKGROUND)

Dengue (DEN) viruses with 4 different serotypes, causing dengue fever (DF), dengue haemorrhagic fever (DHF), and dengue shock syndrome (DSS) are one of medically important arthropod-borne viruses affecting humans in terms of morbidity (1,2). DF/DHF and DSS have re-emerged mainly in tropical and subtropical regions, and the DHF has become the leading cause of death and hospitalization among children in some Southeast Asian countries during the last two decades (3,4).

The principal mosquito vector for the four dengue serotypes is *Ae. aegypti*, a highly domesticated urban species that is well adapted for life in intimate association with humans. Inter-population variation in vector competence of mosquito species for arbovirus has been documented (5,6,7). As for flavivirus, significant variation in oral susceptibility among geographic strains of *Ae. albopictus* and *Ae. aegypti* for the 4 dengue serotypes has been reported (8,9). However the mechanisms responsible for intra and interspecific variation in the ability of Aedes subgenus Stegomyia mosquitoes for infection and transmission of dengue (DEN) viruses are not well understood. Some previous works proposed that it was genetically controlled (8,9), while others considered that the variability of the oral infection rate was attributed to the mesenteronal barrier, since no significant differences were noted when several strains of *Ae. Aegypti* and *Ae. albopictus* were infected by parenteral inoculation (10).

On a worldwide basis, *Ae. aegypti* is the most common vector of yellow fever and dengue fever flaviviruses (11,12,13). Biotypes or geographic races, possibly presenting differences in vectorial capacity, biting behavior, and other traits of epidemiological importance, may be identified using genetic markers (14). Assessment of the genetic relatedness among different geographic populations also provides important evidence to infer vector movement among disease foci or from a site of initial colonization (15).

The susceptibility of mosquitoes to infection with viruses and animal parasites for which they serve as vectors may vary widely among different geographic strains of the same species, and even among individuals of the same strain. It has been demonstrated that *Ae. aegypti* and *Ae. Albopictus* colonies originating from different geographic localities vary in the ability to become infected by the oral route with dengue viruses and that variation is dose related and appears to be genetically controlled (8,9). Whether this reflects difference characteristic of the virus serotype or merely differences between the individual viruses is largely unknown.

2. วัตถุประสงค์ (OBJECTIVES)

2.1 To attempts to better define dengue (DEN) virus infection in its mosquito vector. Furthermore, to ascertain the dynamics of susceptibility and growth analysis of DEN-2 Southeast Asian genotype isolated from patient exhibiting different disease severity in orally infected *Aedes aegypti* originated from different geographic origin, indeed from high and low dengue risk areas.

2.2 To asses the contribution of the genetic structure of *Ae. aegypt*i population to the pattern of dengue fever transmission, by studying the genetic structure and the genetic variation of *Ae. aegypti* collected from high and low dengue risk areas.

3. วิธีการทดลอง (MATERIALS AND METHODS)

3.1 Mosquitoes strains

Four districts (amphoe) in Chachoengsao province, central east of Thailand, with antecedents of dengue cases in the past were selected for the study. Sub-districts (tambon) with high and low incidence of dengue (DF/DHF/DSS) during the past 20 years were selected and classified as High and Low dengue risk areas based on number of houses and inhabitants per square kilometer, and number of cases of dengue annually reported. The locations sampled covered a wide geographical distribution of *Ae. aegypti*, including urban, sub-urban and rural environments. The shortest distance between study areas (districts) was ca. 15 kilometers, and between sub-districts was ca. more than 25 kilometers.

Eight samples of *Ae. Aegypti* were collected between August and September 2002, two from each sub-district (Table 1). Samples from high dengue incidence areas were collected in house with recent report of dengue cases and from low incidence areas in house without dengue cases for the last two years. The mosquitoes were collected as 4^{th} stage larvae and/or pupae, unless 200 in total, from water containers found in the peridomestic habitat and inside of the house, at collection site separated at least ca. 900 mts. away from each other. Field collected mosquitoes (F0 generation) were reared up to adults under room conditions (ca. Temperature $26^{\circ}C \pm 2^{\circ}C$, relative humidity 75% $\pm$ 10%). After morphological identification, adults were kept in cages and females were blood-engorged on mice to produce eggs. The onward generations of mosquitoes were reared in plastic pans (33 cm X 25 cm X 11 cm high approximately) containing 3 liters of aged tap water with a density of 180-210 larvae per container. The

larvae were fed on a diet of mouse food powder and maintained at temperature 26°C ± 2°C, relative humidity 75% ± 10%. Adults were fed on 3% sucrose and females were allowed to mate and then blood-fed on mice to enable egg production. The F0 were stored at -80°C for future genetic analysis. Oral infection experiments it was performed with the females of the first (F1) or second (F2) generations. The strains used for the oral infection experiments and dengue background of the district (ampher) from where the mosquitoes were collected are described in Table 1.

3.2 Virus strains

The four virus isolates were obtained from the sera of patients from Nakorn Ratchasima Provincial Hospital, Northeastern Thailand, diagnosed with DF and DSS in 2002. Clinical diagnosis confirmation and serological assays (ELISA IgG and IgM) was performed at the hospital, while virus isolation and serotype determination were performed by the staff of the Arbovirus Section, National Institute of Health, Medical Science Center, Nonthaburi. The clinical diagnosis and clinical severity grading of each isolate was classified using the World Wealth Organization criteria (WHO, 1986). The serotype was determined as dengue type 2 by reverse transcription-polymerase chain reaction (RT-PCR). Relevant patient information is summarized in Table 2.

All isolates were passage four times from the patient serum before experimentation: for virus isolation, preparation for large-scale culture, and the large scale culture itself (twice). None of the isolate has been purified by any methodology before inoculation into mosquito cells. This was done to ensure that the viral specimens were not altered significantly from their wild type character as found in the host patient.

3.3 Virus assay

A seed virus was prepared by inoculation into a monolayer culture of *Aedes albopictus* clone C6/36 cell line and incubated at 28°C for 8 days in Eagle's medium supplement with 2% heat-inactivated fetal calf serum (FCS) and 0.2 mM each nonessential amino acids. The infected culture fluid was harvested 8 days after inoculation, aliquoted, and stored at –80°C until used. Thus, the virus titer of the isolates was measure by the focus formation test by using BHK-21 cells on 96-well plates, then used for preparation of the infectious meal, blood virus sucrose solution (BVS).

3.4 Oral Infection

To minimize age factors, only 4-5 day-old female mosquitoes was used. About 60 females were placed in cylindrical pint cardboard cages covered at one end with fine non-wettable nylon mesh. These females were deprived of food for 24-36 hrs. prior to the infectious meal, then were allowed to feed in the infectious meal (BVS) consisting of equal volumes of isolate virus suspension, washed rabbit erythrocytes, and 10% sucrose solution. Drops of the infectious meal were placed on the mesh covering the cardboard cage containing mosquitoes as previously described (8,16). Feeding time was limited to 1 hr., fully engorged mosquitoes were collected with an aspirator at 30 minutes intervals and transferred into clean cartons and maintained for up to 14 day at 30 $\pm$ 1°C as a virus external incubation period.

3.5 Detection of virus Infection

Mosquito head and abdomen were severed and homogenized in Trizol LS reagent for total RNA extraction, and then assayed for dengue virus by using RT-PCR method. The detection of RNA viral in the homogenized abdomen was interpreted to indicate the mosquito midgut had become infected. Detection of RNA viral in the homogenized head indicated that the midgut had become infected and that the virus had subsequently disseminated to secondary target organs.

3.5.1 *RNA extraction and RT-PCR*

Total RNA was extracted from head and abdomen by using Trizol LS (Invitrogen) according to the manufacturer's recommendations. Briefly, head and abdomen separately homogenized in 300 µl of Trizol reagent then 99.9 µl of Chloroform were added, and then centrifuged at 14,000 rpm/min for 10 min at 4°C. RNA was precipitated from the aqueous phase (ca. 145 µl) by mixing with 133.2 µl of isopropyl alcohol. Isopropyl alcohol-RNA precipitated was recovered by centrifugation and the RNA pellet was washed once with 90 µl of 75% ethanol followed with a second wash with absolute ethanol and then air dried. The RNA pellet was resuspended in 10 µl of RNase-free water and used as a template in reverse transcriptase PCR (RT-PCR). Synthetic oligonucleotide primer pairs were designed based on published sequence data for dengue virus serotype specific primer, sense (D2-S) and complementary (D2-C) genome nucleotide (nt) regions 1203 to 1222 and 1432 to 1413 respectively (17,18).

Dengue RNA viral was assayed by using one step RT-PCR kit (Quiagen) following the manufacturer's recommendations with slight modifications. Briefly, 2 µl of RNA, and 8 µl of reaction mix, and RNase free water (to a total volume of 25 µl) was reverse transcribe and amplify following the thermal cycle protocol recommended in the kit instruction manual, with some modifications, as follow: one cycle at 53°C for 30 minutes, one cycle at 94°C for 2 minutes, followed by 45 cycles at 94°C for minute, and 53°C for 1 minute, and 68°C for 2 minutes, followed by one cycle at 68°C for 7 minutes. The reaction mix contained a mixture of 5 µl of 5x buffer (contains 12.5 mM $MgCl_2$), 1 µl of dNTP mix (containing 10 mM of each dNTP), 0.5 µl (25 pmol) of corresponding primers, D2-S (5'-GTTCGTCTGCAAACACTCCA-3') and D2-C (5'-GTGTTATTTTGATTTCCTTG-3'), and 1 µl of enzyme mix (an optimized combination of Omnscript Reverse Transciptase, Sensiscript Reverse Transcriptase, and HotStarttTap DNA Polymerase). Nine microliters of PCR product was subjected to agarose gel electrophoresis, and amplified DNA fragments were visualized with ethidium bromide staining.

3.6 Statistical analysis

Homogeneity in the proportions of females infected among *Ae. aegypti* strains between isolate virus and proportions of females of each strains infected by each dengue isolate were compared using the Levene's test of equality, infection cases were weighted for unequal sample sizes. To account for all sources of variation, including variation among individual mosquito strains within a isolate virus, vector infection data was analyzed by analysis of variance and the least significant difference (LSD) was used for the pairwise multiple comparison. Univariate analysis of variance (UNIANOVA) was used to test the effect of mosquito strains, and isolate virus on infection rates. The statistical analysis was done using general linear model univariate analysis (GLM) of SPSS 11.5 software packet.

4. ผลการศึกษา (RESULTS)

Although some mosquito strains were orally challenged on different days they were challenged with the four isolates virus on the same day, all feeding suspensions contain the same virus titer (1×10^2 PFU/ml) were made using an aliquot of the same virus pool and the titers of the post feeding virus suspensions did not significantly changed.

4.1 Screening for mosquito strains susceptibility

The comparative susceptibility of mosquitoes strains to oral infection with four dengue type 2 isolates virus isolated from patients exhibiting different disease severity are summarized in Table 3 and Figure 1. Overall, significantly different disseminated infection rate was observed among mosquito strains ($P < 0.05$), however only TAL-GKE, TAL-RBA, and BPA-GKE mosquito strains pairs were not differentiated on its disseminated infection rates ($P > 0.05$), Figure 1A. Disseminated infection rates for mosquito strain/isolate virus pair ranged from 92 % (TAK/ThNR2/406) to 14.3% (KAO/ThNR2/479), Table 3. The TAK and KKR mosquito strains showed the highest susceptibility, 74% and 71% of 96 mosquitoes respectively. The TAK mosquito strain shown to be more susceptible to isolates virus isolated from patients with DSS than for isolates virus isolated from patients with classical DF symptoms, while KKR, in contrast, was more susceptible to isolates virus isolated from patients with classical dengue fever (DF) than for isolates virus isolated from patients with dengue shock syndrome (DSS) symptoms, Figure 2A. Whereas most of the other mosquito strains showed more less the same susceptibility pattern with an slightly high average disseminated infection rate ranging from 61 - 40 %, , Table 3 and Figure 2A.

To better understand geographical differences the disseminated infection rate was analyzed by grouping the mosquito strains, first, according their habitat origin, rural comprises KKR, GKE, RBK and RBA strains, and urban comprises TAL, KAO, BPA, and TAK strains. The second group, according their geographic origin comprises districts Muang, Bang Pakong, Krong Kruang and Ratchasan. For the first group, even though individual mosquito strains from rural habitat, showed a higher disseminated infection rate (92 – 18 %) than those for strains from urban habitat (88 – 14.3 %), Table 3. However, overall, mosquito strains from urban habitat were significantly more susceptible (62.3 % of 416 mosquitoes) than strains from rural habitat (55.8% of 493 mosquitoes), $P < 0.05$, Figure 1B. On the other hand, when isolate virus were grouping according the disease severity of the patient from who the virus was isolated, mosquitoes from rural habitat showed to be more significantly susceptible to isolates virus isolated from a patient with DF symptoms than that isolate virus isolated from a patient with DSS symptoms. Whereas mosquito strains from urban habitat were undifferentiated susceptible to the isolate virus isolated either from a DF patient or from a DSS patient, Figure2B. For the second group, districts, the disseminated infection rates were heterogeneous among districts, $P < 0.05$. The mosquito strains from districts

Bang Pakong (67.2% of 204 mosquitoes) and Krong Kruang (63.5% of 153 mosquitoes) showed the highest susceptibility. Whereas mosquito strains from districts Muang (57.5% of 212 mosquitoes) and Ratchasan (48.4% of 252 mosquitoes) showed to be less susceptible, Figure 1C. Mosquito strains from districts Muang and Krong Kruang shown to be more susceptible to the infection with virus isolated from DF patient than that with virus isolated from DSS patient. While mosquito strains from Bang Pakong and Ratchasan districts were more susceptible to the infection with virus isolated from DSS patient than that with virus isolated from DF patient, Figure 2C.

4.2 Ascertaining isolates virus infectivity

Variation on the proportion of infected mosquitoes was observed among isolates virus. The proportion of mosquitoes infected by isolates virus isolated from patients exhibiting mild disease symptoms, classical dengue fever (DF), 59.7 % of 477, was significantly higher that that infected by isolates virus isolated from patients exhibiting severe disease symptoms, dengue shock syndrome (DSS), 57.6 % of 432 mosquitoes, $P < 0.05$, Figure 1D. Furthermore, variation on the proportion of mosquitoes infected by individual isolate virus was observed. The proportion of mosquitoes infected by isolate virus ThNR2/772 (63.4% of 238) and ThNR2/406 (56% of 239) was significantly higher than that the infected by ThNR2/391 (60.4% of 238) and ThNR2/479(55.1% of 238), $P < 0.05$, Figure 1E. However, the proportion of infected mosquitoes for individual isolate virus within the same disease severity was not statistically significant different ($P > 0.05$), Figure 1E.

On the other hand, when the mosquito strains were grouping according their origin significant variation was observed on the proportion of infected mosquitoes for individual isolate virus between habitats, Figure 3. The proportion of mosquitoes infected by ThNR2/772, ThNR2/391, and ThNR2/479 isolate virus was significantly higher for mosquito strains from urban habitat than that for mosquito strains from rural habitat ($P < 0.05$), whereas the proportion of mosquitoes infected by ThNR2/406 isolate virus, in contrast, was higher for mosquito strains originated from rural habitat than that from urban habitat, Figure 3A. Furthermore, variation on the proportion of infected mosquitoes for individual isolate virus was observed among the different districts. The isolates ThNR2/406 and ThNR2/772 were significantly more infective for mosquito strains from Krong Kruang and Bang Pakong district, respectively, and less infective for mosquitoes from Ratchasan. Whereas, the isolates ThNR2/391 and ThNR2/479 showed

to be significantly more infective for mosquito strains from Bang Pakong and less infective for mosquitoes from Muang, Figure 3B.

5 บทวิจารณ์ (DISCUSSION)

Oral susceptibility to experimental infection with all 4 serotypes of dengue virus has been studied for *Aedes* sp. and strains of a given species (5-8,19) and more recently, variation in susceptibility to oral infection with DEN-2 virus in *Ae. aegypti* from different geographic origin has been reported (20–24). Differences on the proportion of infected mosquitoes between DEN-2 virus strains SE and American genotype has also been reported in geographically separated *Ae. aegypti* populations (25-27). In contrast to previous studies, in the present study we used low-passage isolates virus recently isolated from patients exhibiting different disease severity, dengue fever (DF) and dengue shock syndrome (DSS), respectively, which are sympatric to the *Ae. aegypti* strains used. We found significant variation on disseminated infection rate in *Ae. aegypti* according mosquito strains, habitat and geographic origin, virus isolates, and patient disease severity. Furthermore, significant variation on the proportion of mosquitoes infected by individual virus strains were found, as well as between virus isolated from patients exhibiting dengue fever and dengue shock syndrome disease severity.

The susceptibility of *Ae. aegypti* to infection by dengue virus is determined by an array of vector, viral, and environmental factors. In the present study, to minimize the effect of environmental variation, we challenged each mosquito strains with all 4 isolates of dengue virus at the same time, and mosquitoes were held together at the same environmental fluctuations during the incubation period (14 days). Even though all mosquito strains may not experienced the same environmental condition not statistical significant variation was observed when it was compared the temperature and humidity mean variation that each mosquito strain experienced during the incubation period (data not shown). Nonetheless, some of mosquito susceptibility variation we observed may have been influenced by environmental variation among experimental groups. Overall, mosquito strains from urban habitat were more susceptible than strains from rural habitat, however some mosquito strain from urban and rural habitat have undifferentiated infection rates, namely TAL with GKE and RBA (from urban and rural, respectively), and BPA with GKE (Figure 1B). It is interesting that, even though was observed a significant difference in the susceptibility to dengue virus between mosquito strains from urban and rural habitat, as well as between districts, the geographic origin

of the mosquito strains, *per se*, is not necessarily indicative of susceptibility status of the *Ae. aegypti*. This can be seen in mosquito strains from the same district, which are geographically close ca. 3 to 5 Kms., have significantly different infection rates (Figure 1A). A detailed genetic analysis is needed to determine if these mosquito strains are actually isolated and constitute independent populations that differ in susceptibility to the infection with dengue virus. In previous studies, it has been described that mosquito from most populated and urbanized areas are genetically highly differentiated and display high and heterogeneous infection rate and this genetic differentiation has been related to the intensity of insecticide control and human population (22,24).

This study also raised the possibility that the susceptibility variation is not randomly distributed across the disease severity of the patient from whom the virus was isolated. Specifically, mosquitoes were more susceptible to virus isolated from patients exhibiting mild symptoms, classical dengue fever, than virus isolated from patients exhibiting severe symptoms, dengue shock syndrome (Figure 1D), however the results show no correlation between mosquito susceptibility and disease severity. *In Vitro* analysis of DEN-2 viruses isolated from patients exhibiting different disease severity also reported no correlation between infectivity and cell destruction capability of the virus (28), and virus infectivity titers in the culture fluids of the C6/36 cells did not correlate with the severity of the disease of the patient from who each isolate was isolated (29). On the other hand, we speculate that some mosquito vector may become adapted to the circulating virus in a given area. Indeed, we found that mosquito strains from district with high incidence of dengue cases (Bang Pakong, urban habitat) was significant more susceptible that the other mosquito strains. However, the evidence that mosquito strains from Krong Kruang district (rural habitat, low dengue incidence) were more susceptible than that strains from Muang district, though have lower incidence of dengue cases than Muang, argues against the possible local adaptation.

Dengue viruses exhibit a considerable variation in their efficiency to infect and dissemination in its vector mosquitoes. In the present study, we evaluated virus infectivity by measure the proportion of mosquitoes infected by individual virus isolate. Overall, we found variation in the proportion of mosquitoes infected by individual virus isolate. Specifically, the ThNR2/406 and ThNR2/772 isolates shown to be more infective than the ThNR2/391 and ThNR2/479 isolates viruses. However, the isolate virus infectivity is seems like to be differentiated according mosquito strain-virus isolate pairs (Figure 3). Relatively few genetic differences among dengue virus isolates may have

significant effect in the infection, dissemination of and transmission by vector mosquitoes. Previous studies on DEN-2 viruses revealed differences in the proportion of mosquitoes infected by individual virus isolates and have been associated to molecular differences among isolate virus (30,25-27). Thus, we speculate that differences in the proportion of infected mosquitoes by individual isolate virus are probably due to certain genetic elements of the isolate virus.

In this study, we analyzed 32 recently collected virus-vector pairs (eight geographically different mosquito strains versus four isolate virus strains). In all virus-vector pairs the infection rate were different, moreover many of those were significantly different. This would allow us to conclude that the variation of the susceptibility among mosquito strains is probably due to the combination of certain genetic elements of the vector and the virus. Furthermore, these results suggested that the efficacy of dengue virus circulation in a given locale it could be varied according to interaction of virus strains and origin of the vector mosquitoes. These studies lay the groundwork for ascertain the role of the association of genetic elements of mosquito determinants of susceptibility and the genetic variations of dengue virus in the occurrence of different disease severity.

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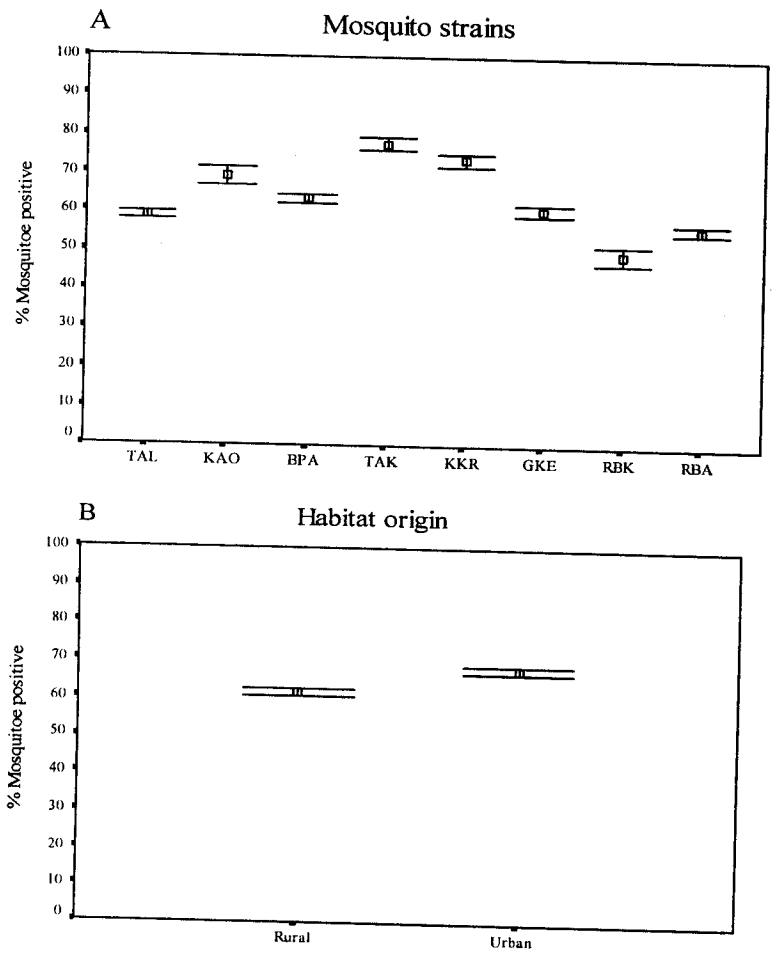
7. ผลงานที่ได้จากงานวิจัย (Output)

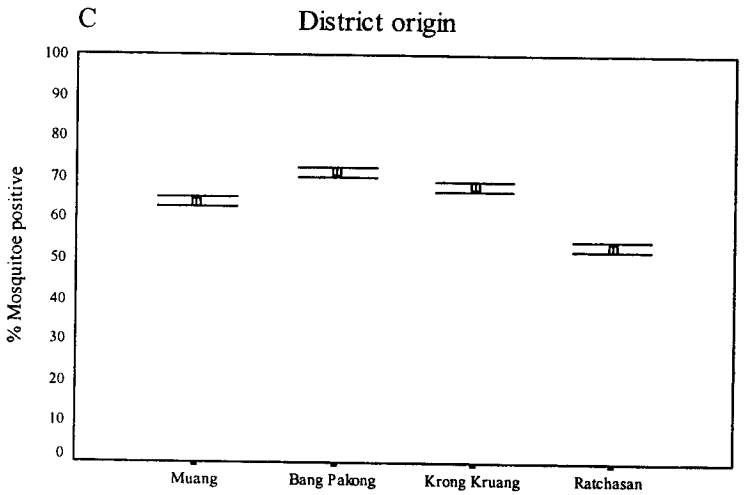
กำลังอยู่ในช่วงเตรียมผลงานเพื่อตีพิมพ์ในวารสารระดับนานาชาติ,

8. ภาคผนวก

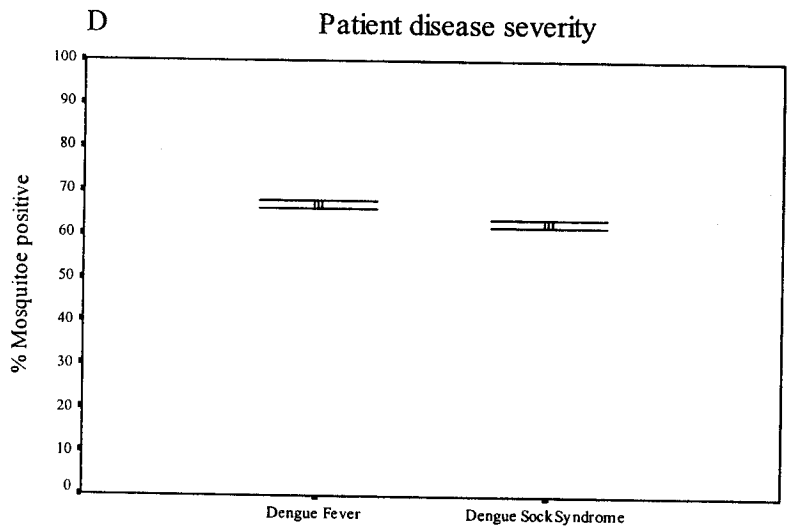
Figure 1 Disseminated infection rates of four dengue type 2 isolates virus in *Aedes aegypti* according **A**, mosquito strain, and **B**, habitat origin, **C**, district origin, **D**, patient disease severity, and **E**, isolate virus. Error bars shown the mean ± 95% confidence level.



C


District origin
% Mosquitoe positive
100
90
80
70
60
50
40
30
20
10
0
Muang
Bang Pakong
Krong Kruang
Ratchasan

D


Patient disease severity
% Mosquitoe positive
100
90
80
70
60
50
40
30
20
10
0
Dengue Fever
Dengue Sock Syndrome

E

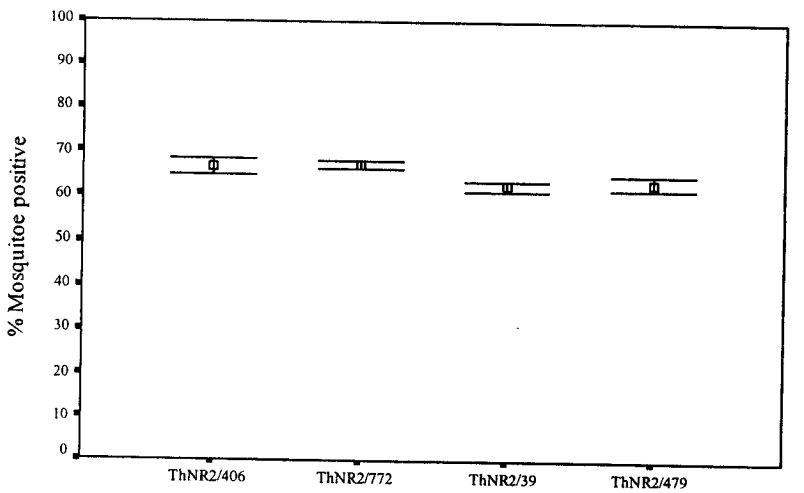

Isolate virus
% Mosquitoe positive
100
90
80
70
60
50
40
30
20
10
0
ThNR2/406
ThNR2/772
ThNR2/39
ThNR2/479

Figure 2 Comparative oral susceptibility to dengue type 2 virus isolated from patients exhibiting different disease severity in orally infected *Aedes aegypti* according **A**, mosquito strain, **B**, habitat origin, **C**, district origin. Viruses isolated from patients exhibiting dengue fever (DF) severity are depicted by a filled square. Viruses isolated from patients exhibiting dengue shock syndrome (DSS) disease severity are depicted by a open circle. Error bars shown the mean ± 95% confidence level.

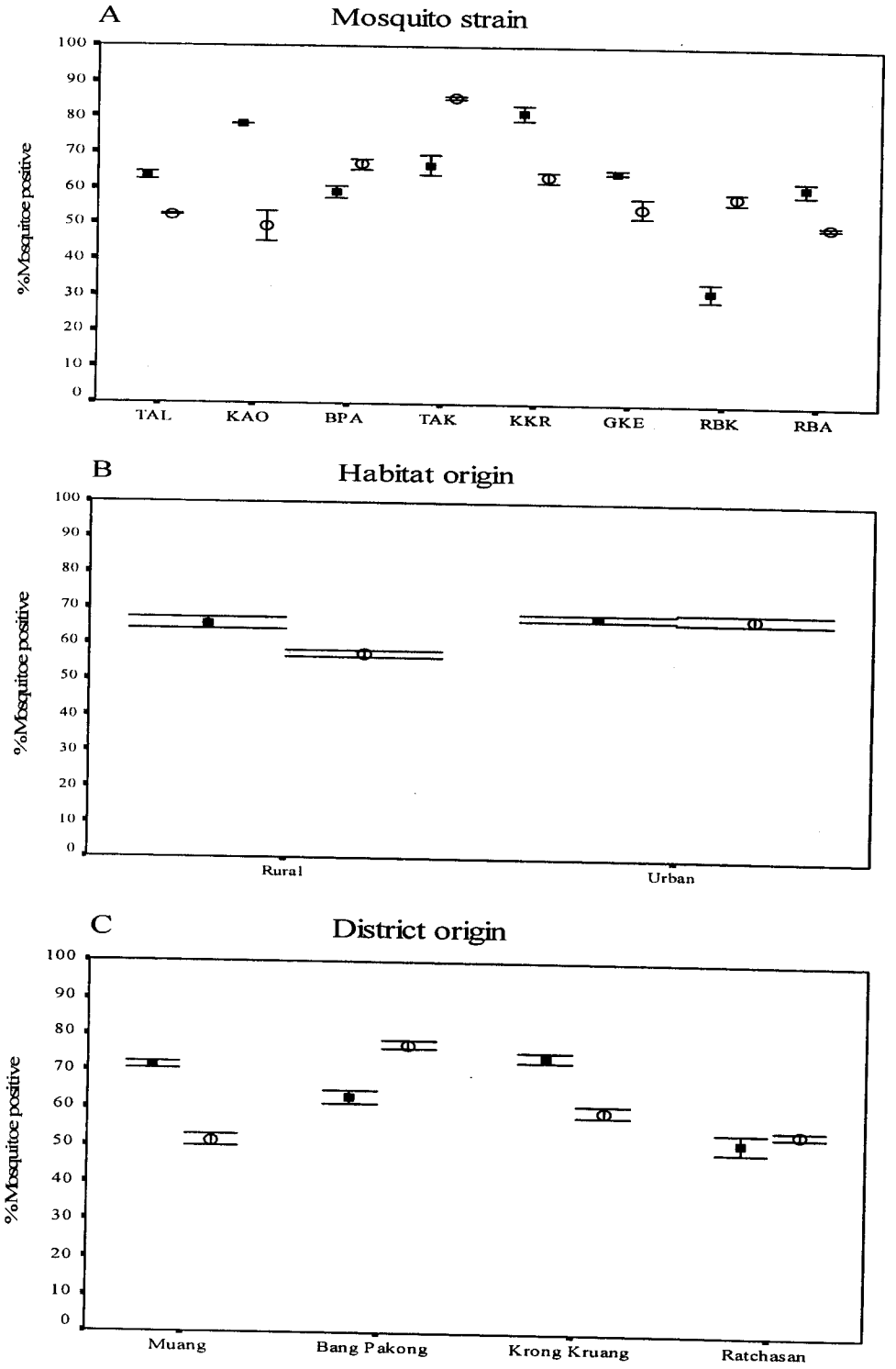


Figure 3 Proportion of *Aedes aegypti* mosquitoes infected by four isolates of dengue type 2 virus according **A**, habitat origin, **B**, district origin, and **C**, mosquito strains.

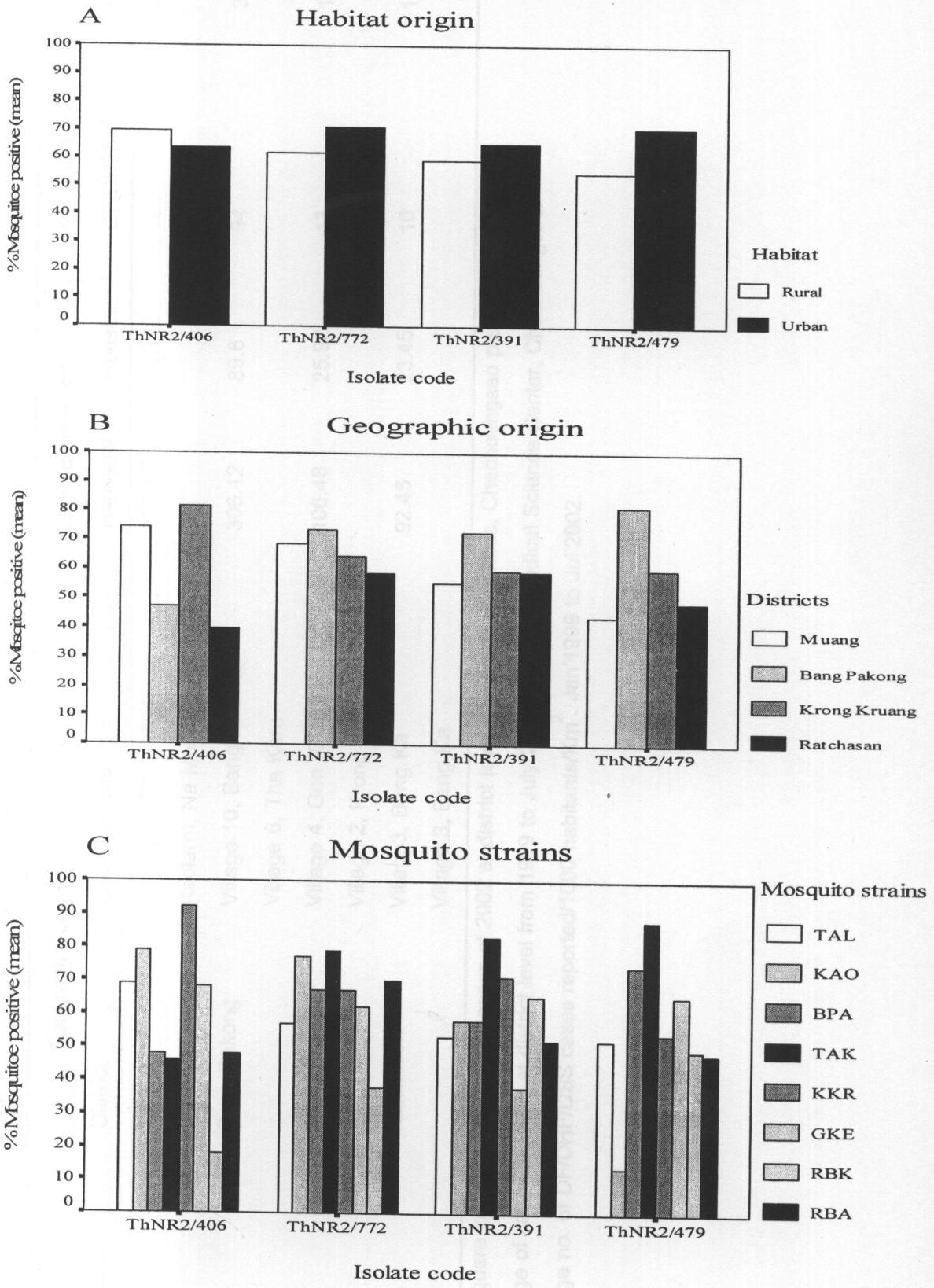


Table 1. Demographic information and dengue (DEN) background of the geographic origin of *Aedes aegypti* strains used.

Mosquito strain code	Geographic origin		Demographic data[a]		Dengue background[b]	
	District	Collection site	Habitants	Houses	DF/DHF/DSS	Incidence[c]
TAL	Muang	Taladbon, Na Muang	369.17	106.83	183	495.7
KAO		Kaolarm, Na Muang				
BPA	Bang Pakong	Village 10, Bang Pakong	306.12	89.6	94	307.1
TAK		Village 6, Tha Kam				
GKE	Krong Kruang	Village 4, Gon Kheo	106.48	25.98	13	122.1
KKR		Village 2, Krong Kruang				
RBK	Ratchasan	Village3, Bang Ka	92.45	23.45	10	108.2
RBA		Village 3, Bang Ka				

[a] Per square kilometer, based on census of 2002 at district level, Public Health Office, Chachoengsao province.

[b] Average of cases reported at district level from 1999 to July 2002, Regional Medical Science Center, Chachoengsao.

[c] Average no. of DF/DHF/DSS cases reported/1000 habitants/Km^2. Jan'1999 to Jul'2002.

Table 2. Medical data and *in Vitro* Infectivity results of four of DEN-2 isolates virus used from Nakorn Ratchasima province, Northeast of Thailand.

	Medical data						
			Clinical	Antibody response	ELISA assay (unit)		Infectivity
Isolate name	Sex	Age	Diagnosis	Pattern	DEN-IgG	DEN-IgM	LLC-MK2 (PFU/ml)
ThNR02/406	M	16	DF	Uncharacterized [A]	7	0	1.2×10^5
ThNR02/772	M	14	DF	Uncharacterized	14	9	8.5×10^3
ThNR02/391	M	12	DSS	Uncharacterized	29	2	1.38×10^5
ThNR02/479	M	12	DSS	Secondary	3	0	1.0×10^2
					149 [B]	56	

[A] Due the lack of a second serum sample.

[B] Second serum sample was obtained 2 weeks after the first sample.

Table 3. Disseminated infection rates* toward a four dengue type 2 isolates virus in orally challenged *Aedes aegypti* from Chachoengsao province, Central East of Thailand.

Strain	Dengue Fever (DF)		Dengue Shock Syndrome (DSS)	
	ThNR2/406	ThNR2/772	ThNR2/391	ThNR2/479
TAL	24/35 ^{Aa}	19/33 ^{Ba}	16/30 ^{Ca}	17/33 ^{BCa}
KAO	15/19 ^{Ab}	17/22 ^{ACbd}	11/19 ^{Bb}	3/21 ^{Cab}
BPA	14/29 ^{Ab}	24/36 ^{Bc}	14/24 ^{Cc}	14/19 ^{Cc}
TAK	11/24 ^{Ab}	19/24 ^{Bab}	20/24 ^{Bd}	21/24 ^{Bd}
KKR	22/24 ^{Aa}	16/24 ^{Bd}	17/24 ^{Bac}	13/24 ^{Bc}
GKE	27/40 ^{Aa}	23/37 ^{Bc}	13/34 ^{Bd}	22/34 ^{Bd}
RBK	7/39 ^{Ac}	12/32 ^{Ba}	15/23 ^{Cc}	20/41 ^{D}
RBA	14/29 ^{Ab}	21/30 ^{Bac}	19/29 ^{ABad}	14/29 ^{Cb}

* Number of mosquitoes positive for dengue type 2 RNA viral in head tissues/number tested.

ANOVA (LSD) values in the same row with the same capital letter (A or B or C or D) do not show a significant difference ($P>0.05$). Those in the same column and with the same lower case letter (a or b or c or d) do not show a significant difference ($P>0.05$).

Table 4. Comparisons of disseminated infection rates according the origin of mosquitoes, mosquito strain, and disease severity and isolate virus in orally challenged *Aedes aegypti* from Chachoengsao province, Central East of Thailand.

Variation	N	Probability of homogeneity*	
		F *value*	P *value*
Mosquito origin			
Habitat	2	29.838	< 0.05
Urban	4	9.813	< 0.05
Rural	4	13.074	< 0.05
Geographic	4	13.796	< 0.05
Muang	2	15.467	< 0.05
Bang Pakong	2	6.551	< 0.05
Krong Kruang	2	29.592	< 0.05
Ratchasan	2	26.855	< 0.05
Mosquitoes strain	8	11.092	< 0.05
Disease severity	2	67.465	< 0.05
Isolate virus	2	66.171	< 0.05

*Levene test of the homogeneity of variance.

Table 5. ANOVA test for effects of mosquito strain and isolate virus on disseminated infection rates in orally challenged *Aedes aegypti* from Chachoengsao province, Central East of Thailand.

Source variables	df	F-value	P-value
Mosquitoes strain [a]	7	28.317	< 0.05
Isolates virus [b]	3	23.635	< 0.05
Mosquito strain x Isolate virus	21	14.294	< 0.05

[a] Refer to table 1 for description. [b] Refer to Table 2. x = Interaction.