A SURVEY OF DENGUE VIRAL INFECTION IN *AEDES AEGYPTI* AND *AEDES ALBOPICTUS* FROM RE-EPIDEMIC AREAS IN THE NORTH OF THAILAND USING NUCLEIC ACID SEQUENCE BASED AMPLIFICATION ASSAY

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Abstract. Immature stages of *Aedes aegypti* and *Ae. albopictus* were collected from 17 dengue re-epidemic areas in Chiang Mai and Lampang Provinces, in the north of Thailand. They were reared to adults and tested for dengue viral RNA by a nucleic acid sequence based amplification assay (NASBA). Of a total of 9,825 *Ae. aegypti* and 150 *Ae. albopictus* examined, none of them were found positive for the virus, suggesting that transovarial transmission may be very low in the vector populations and may not play a significant role in the epidemiology of dengue infection in Thailand.

INTRODUCTION

In Thailand, the first outbreak of dengue hemorrhagic fever (DHF) was recognized in 1958 with 2,500 cases in Bangkok metropolitan area. Since then its incidence has increased cyclically (Charoensuk *et al*, 1999). Several factors play into its complex epidemiology, including mosquito, virus, man, climate and environment (WHO, 1997). *Aedes aegypti* (L) and *Aedes albopictus* (Skuse) are regarded as the primary and secondary vectors in Thailand. At present, the patterns of outbreaks appear different from the past five decades, and thus are more difficult to predict (Charoensuk *et al*, 1999; Cummings *et al,* 2004). There are many areas that have reported experiences of re-epidemics of DHF with a high incidence in the dry season (MoPH, 2005). The interactions between susceptible vectors and the virus have a direct effect on the introduction and subsequent maintenance of dengue infection in a particular community (WHO, 1997). However, it is still unclear where and how the viruses persist in nature in the absence of viremic vertebrate hosts or other unfavorable conditions for mosquito activity. In transovarial transmission, the virus is transmitted vertically from an infected female to her offspring, and in venereal transmission, an infected male transmits the virus to the female during copulation. Both are considered to play important role in the maintenance of virus in the vector population as reported by several authors (Khin and Khin, 1983; Rosen *et al,* 1983; Hull *et al,* 1984; Rosen, 1987; Mitchell and Miller, 1990; Joshi *et al,* 1996; Gokhale *et al,* 2000; Thenmozil *et al,* 2000; Joshi and Sharma, 2001). Several studies have demonstrated the vertical transmission of dengue

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virus in the laboratory by either injection or oral infection to mosquitoes for several generations (Mitchells and Miller, 1990; Joshi *et al,* 2002; Vazeille *et al,* 2003). The probability of detecting vertical transmission is generally low and appears to be geographically specific to species and strains of mosquitoes and dengue serotypes (Rosen *et al*,1983; Thenmezhi *et al*, 2000). Nonetheless, this information may be useful for an early warning surveillance system and planning for DHF control. To date, little is known about the transovarial transmission of dengue virus in the mosquito vectors in Thailand. In this study, a nucleic acid sequence-based amplification assay (NASBA) was used to determine the dengue virus in wild populations of *Ae. aegypti* and *Ae. albopictus* reared from their immature stages.

MATERIALS AND METHODS

Study sites

Villages in Chiang Mai and Lampang Provinces in northern Thailand where dengue cases have been reported continuously or where re-epidemics have occurred during the past two years, 2003 and 2004, were selected as the study sites. A total of 7 localities in Chiang Mai and 10 in Lampang were chosen (Table 1).

Mosquito collection

Third- and fourth-instar larvae and pupae of *Aedes* mosquitoes were collected from artificial and natural containers in the selected localities during April (dry season) and May-August 2005 (wet season). All specimens were transferred to the insectarium of the Vector

Table 1
Aedes aegypti and *Ae. albopictus* collected from each locality in Chiang Mai and Lampang Provinces in 2005.

Province		Locality	Dry season				Wet season				Total
			Aedes aegypti		*Ae. albopictus*		*Aedes aegypti*		*Ae. albopictus*		
			Male	Female	Male	Female	Male	Female	Male	Female	
Chiang Mai	1	Chang Pauw M	20	22	0	0	33	19	0	0	94
	2	Wat Ket M	38	18	0	0	35	53	0	0	144
	3	Haiy Ya M	60	118	0	0	174	152	0	0	504
	4	Sri Phum M	24	19	0	0	99	56	0	0	198
	5	Suthep M	23	41	0	0	35	60	0	0	159
	6	Suthep canton	19	58	0	0	19	24	0	0	120
	7	Chang Pauw canton	17	25	0	0	17	19	0	0	78
Lampang	1	Sop Toui M	40	100	0	0	197	202	0	0	539
	2	Phar Baddh M	37	17	0	0	933	727	40	37	1,791
	3	Vieng Nua M	20	18	0	0	220	284	0	0	542
	4	Hou Vieng M	118	160	0	0	480	417	0	0	1,175
	5	V. 1 Toung Fai	20	20	0	0	632	553	0	0	1,225
	6	V. 3 Toung Fai	159	158	0	0	248	160	17	16	758
	7	V. 4 Toung Fai	237	140	0	0	365	374	0	0	1,116
	8	V. 7 Lom Lad	22	24	0	0	309	259	0	0	614
	9	V. 11 Sadet	18	19	0	0	394	357	0	0	788
	10	V. 3 Vieng Tal	26	21	0	0	24	19	23	17	130
Total			898	978	0	0	4,214	3,735	80	70	9,975

V = village, M = municipality areas

Borne Disease Section, Office of Disease Prevention and Control No. 10 , Chiang Mai. Pupae were separated and kept in a 30 cm^3 mosquito cage, while larvae were reared in plastic trays and provided with a ground dog biscuit twice a day. Ten percent sucrose solution was provided to the newly emerging adults. After morphological identification of the adults, *Aedes aegypti* and *Ae. albopictus* from each locality were kept separately by sex. Each group was then pooled (10-20) and stored at -70°C until the virus assay was performed.

Dengue viruses and negative control virus

Prototypes of dengue serotype seeds, dengue 1(Hawaii), dengue 2 (NEW Guinea C), dengue 3 (H87) and dengue 4 (H241) were used to spike five female adult *Ae. aegypti* and *Ae. albopictus* mosquitoes (laboratory strains) in each of 100 μl of serotype-specific positive control, which were obtained from Mahidol University (Usawattanakul *et al*, 2002a,b; Ratanasetyuth, 2004). Negative controls were prepared from five female *Ae. aegypti* and *Ae. albopictus* mosquitoes in 100 μl of NucliSens NASBA water (bioMerieux bv, Boxtel, The Netherlands).

RNA extraction

Mosquito samples were extruded by squeezing and mixing with 900 μl of lysis buffer (5 M guanidine thiocyanate, 1.2% (w/v) Triton X-100, 0.1 M Tris/HCl pH 6.4, 0.2 M EDTA). Nucleic acid extraction method was based on the method of Boom *et al* (1990). In brief, the sample preparation step involves the release and stabilization of all nucleic acid by guanidine thiocyanate lysis, and under high salt conditions, nucleic acid will be bound to the silica particles. Several washing steps remove amplification inhibitors before the nucleic acid is eluded. Final nucleic acid extracts were obtained in a total of 50 μl of elution buffer (1mM Tris/HCl pH 8.0, 1 mM EDTA) and transferred to new microtubes. Amplification procedure was performed within an hour.

RNA amplification

Extracted dengue viral RNA was amplified by NASBA method according to the manufacturer's instructions (bioMerieux bv, Boxtel, The Netherlands). NASBA is an isothermal amplification process (41°C) in which the reactions (including reverse transcription) occur simultaneously in a single tube. Five μl of extracted RNA template was produced up to a 20 μl final reaction mixture containing 10 μl of 40 mM Tris/HCl (pH 8.5), 45% dimethylsulfoxide(DMSO), NASBA water, 80 mM KCl, Primer 1 (25 μM Dengue P1), Primer 2 (25 μM Dengue P2), 10 μM RNA Dengue detector probe, nucleotides, 5 mM dithiothreitol and 12 mM $MgCl_2$ and 5 μl of working enzyme (1.5M sorbitol in aqueous solution, 6.4 U AMV-RT, 0.1 U RNase H, 32 U T7-RNA polymerase and 0.1 μg of BSA/μl). The amplification product was analyzed within 24 hours or stored at -20°C for no more than 30 days before analysis. Sequences of dengue primers P1 and P2 and detector probe are listed in Table 2.

RNA detection

Amplification product was detected using an electrochemiluminescent (ECL). ECL is the chemiluminescent reaction of species that are generated electrochemically at the surface of

Table 2
Primers and probe sequences used in the detection of dengue virus by NASBA assay.

	Sequence (5 to 3)	Position
Primer P1	AAT TCT AAT ACG ACT CAC TAT AGG GGA GAC A	10,632-10,653
Primer P2	GAT GCA AGG TCG CAT ATG AGG GTT AGA GGA G	10,551-10,520
Detector probe	CCC AGC AAA CAG CAT ATT GAC GCT GGG	10,615-10,635

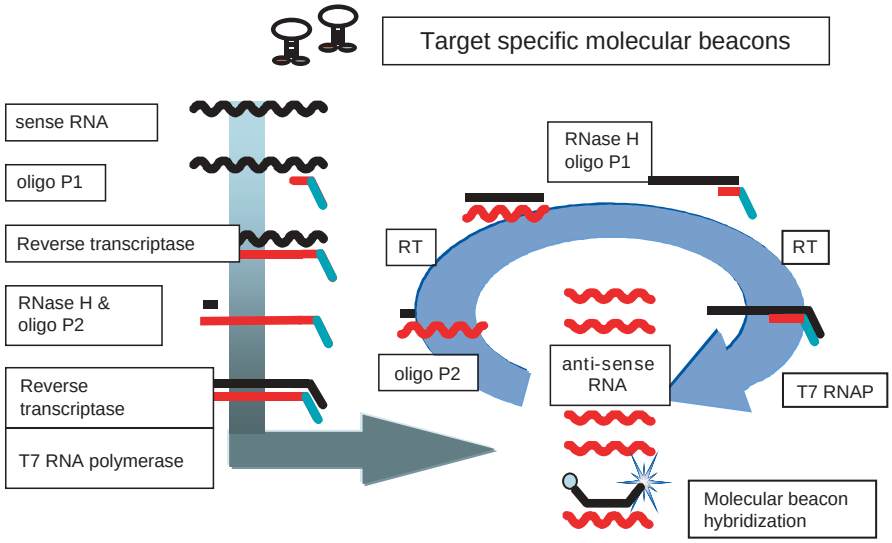


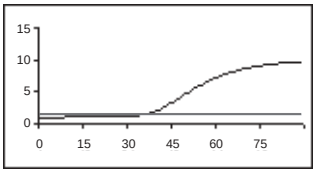
Fig 1–Diagram of location of primers, probe on the virus RNA and the Real-time detection in NASBA. NASBA is achieved using the P1(antisense)-P2 (sense) oligonucleotide set. The overhang on P1 encodes the promoter sequence for the T7 RNA polymerase; the overhang on P2 is a generic ECL detection probe sequence that is homologous to the ruthenium-labeled detected probe. The four serotype-specific capture probes and the conserved sequence capture probe are all in the sense orientation and are immobilized onto the surface of magnetic bead.

an electrode according to the manufacture's instructions (bioMerieux bv, Boxtel, The Netherlands). The analyzer reads fluorescence from reaction tubes in a temperature-controlled environment using an advanced optical system on direct and focused illumination. Dengue Primer 2 contains not only a sequence identical to the target RNA but also a 5´ stretch of 20 nucleotides unrelated to the target RNA. The generated RNA products (amplicons) will contain this unique sequence (referred to as the ECL tail). Amplicons were hybridized to a target-specific probe (coupled to paramagnetic beads) and a generic electrochemiluminescent (primer generic for all dengue serotype but not primer specific for either) probe complementary to the ECL tail. Following hybridization, the bead/amplicon/ECL probe complexes were captured at the magnet electrode of the automated ECL reader (Fig 1). Subsequently, a voltage pulse triggered the ECl reaction. The reaction was conducted at 41°C for 90 minutes. Measured ECL counts were processed and validated by the Basic Kit user software. The user-defined level and control criteria validated every assay run and each individual sample result.

RESULTS

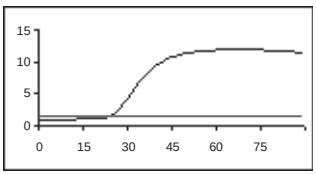
From 17 localities of re-epidemics areas during the years 2003 and 2004 in Chiang Mai and Lumpang Provinces, a total of 9,975 immature *Aedes* mosquitoes were collected in the dry and rainy seasons, and were reared to adulthood and screened for dengue virus (Table 1). Of these, 9,825 and 150 mosquitoes samples were *Aedes aegypti* and *Ae. albopictus,* respectively. The ECL cutoff value for each test (mosquito pool) was derived from all experimental pools. The highest and lowest values were deleted and the arithmetic mean ($\bar{\chi}$) was calculated from the remaining values of the test. The ELC values of individual positive controls were always higher than cut-

DEN-1



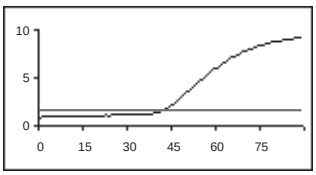
WT signal:	9.698
Signal detected	Δ:8.699
SC signal:	1.000
Signal detected	Δ:0.000

DEN-2



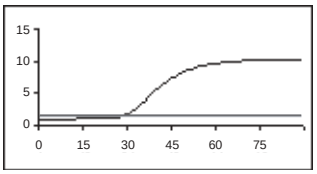
WT signal:	11.377
Signal detected	Δ:10.378
SC signal:	1.000
Signal detected	Δ: 0.000

DEN-3



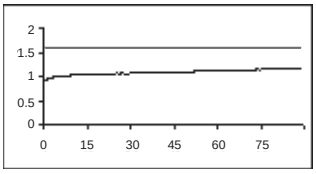
WT signal:	9.193
Signal detected	Δ: 8.193
SC signal:	1.000
Signal detected	Δ: 0.001

DEN-4



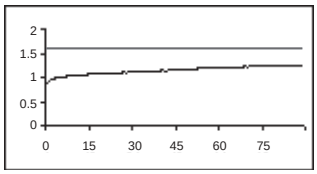
WT signal:	10.258
Signal detected	Δ:9.258
SC signal:	1.000
Signal detected	Δ:0.000

Aedes albopictus



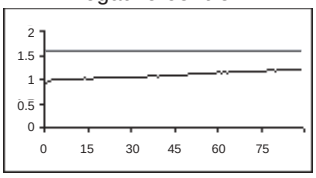
WT signal:	1.173
Signal detected	Δ:0.173
SC signal:	1.000
Signal detected	Δ:0.000

Aedes aegypti



WT signal:	1.238
Signal detected	Δ:0.239
SC signal:	1.000
Signal detected	Δ: 0.000

Negative control



WT signal:	1.204
Signal detected	Δ:0.204
SC signal:	1.000
Signal detected	Δ:0.000

Fig 2–Typical NASBA results of pool mosquito samples, *Aedes aegypti* and *Ae. albopictus* determined by the ECL reader/ Basic Kit user software (www.nuclisens.com). x and y axis show mean time to positive in minutes and normalized fluorescence respectively. Sample positive for Dengue virus = WT (wild type) signal detected >Δ 1.0; negative for dengue virus = WT signal detected< Δ 1.0.

off values (9-11) (Fig 2). None of mosquito sample pools were found positive for dengue virus.

DISCUSSION

In this study, the NASBA assay was used to detect dengue viral RNA from mosquitoes collected from re-epidemic dengue areas in Chiang Mai and Lampang Provinces in 2005. This assay was chosen as it has been shown to be specific to dengue virus, with no-cross reaction with other flaviviruses or non-dengue related viruses (Wu *et al,* 2001; Usawattanakul *et al*, 2002a,b). It is capable of detecting 1PFU/ml of four dengue serotypes (Usawattanakul *et al*, 2002a,b). In addition, it is a rapid technique in a closed system thereby avoiding contamination and does not need a thermal cycler. A recent study in Nong Khai Province, northeast Thailand, succeeded in detecting dengue viral RNA with a viral infection rate of 1.61% of DEN-1 and DEN-2 in field caught *Ae.aegypti* female mosquitoes in the rainy season (Ratanasetyuth, 2004). However our study failed to detect dengue virus in 9,975 mosquitoes, mostly *Ae. aegypti*. The number of *Ae. albopictus* examined in this study was very low because most collections were performed in urban areas where *Ae. aegypti* predominated. Previously Watts *et al (*1985) using a direct fluorescent antibody technique and a

plaque reduction neutralization test to detect natural transovarial transmission of dengue viruses by *Ae. aegygpti* and *Ae. albopictus* in Bangkok, could isolate the virus from 14 of 268 wild caught *Ae. aegypti* female, but failed to detect dengue virus in wild caught larvae, pupae, and male of *Ae. aegypti* (about 6,000 specimens) and *Ae. albopictus* (about 3,000), including adults reared from immature stages.

Field investigations conducted in Myanmar (Khin and Khin, 1983), Trinidad (Hull *et al*, 1984), India (Thenmozhi *et al*, 2000) and Singapore (Kow *et al*, 2001), showed that transovarial transmission of dengue viruses occurred naturally in *Ae. aegypti* and *Ae. albopictus*. However, the viral infection rates were generally low from less than 0.01% to 2.15%. The failure to detect transovarial transmission by *Ae. aegypti* and *Ae. albopictus* in this and recent studies suggests that in nature this mode of transmission may be very low (if any) in the Thai vector populations. It is also suggested that transovarial transmission may not play an important role in maintenance of dengue virus in vector populations in Thailand and can not be used as an early indicator of a dengue outbreak.

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