RAPID DETECTION OF DENGUE VIRAL RNA IN MOSQUITOES BY NUCLEIC ACID-SEQUENCE BASED AMPLIFICATION (NASBA)

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Abstract. RNA amplification by nucleic acid sequence-based amplification (NASBA) was used to detect serotype specific dengue viruses in artificially-infected female *Aedes* mosquitoes, in comparison with RT-PCR technique. NASBA could detect dengue virus serotype 2 and 4 below 0.1 PFU, which was more sensitive than RT-PCR, but this technique was as sensitive as RT-PCR when detecting dengue virus serotype 1 and 3. Dengue viruses could be detected at the thorax of mosquitoes at 0, 7, and 14 days after inoculation with dengue virus serotype 2. This method should be useful for virological surveillance of dengue infected *Aedes* mosquitoes, as an early warning system to predict outbreaks of dengue viruses.

INTRODUCTION

Dengue virus is the most important arthropod-borne viral disease of public health significance. Its geographic distribution includes more than 100 countries worldwide, where more than 2.5 billion people are at risk of dengue infection and there are an estimated 50 million infections per year. The major disease burden is found in Southeast Asia and the Western Pacific (WHO, 1999; Gubler, 2002). In 2002, this disease was in the top 10 for morbidity and mortality in Thailand (Bureau of Epidemiology, 2003).

Dengue virus infection may be asymptomatic or may lead to undifferentiated fever (viral syndrome), dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). These diseases are caused by four dengue viruses (DENV-1, DENV-2, DENV-3, and DENV-4), which are antigenically distinct but have the same epidemiology (WHO, 1997; Gubler, 2002). *Aedes (Ae.) aegypti* is considered the main vector because this species is closely associated with human habitation, but in some regions other *Aedes* species, such as *Ae. albopictus* and *Ae. polynesiensis*, are also involved. Transovarial transmission is considered to be important in maintaining dengue virus at a low level of transmission within the human population (Rigau-Perez *et al*, 1998).

Although dengue virus infection leads to pediatric hospitalization and death, a decrease in incidence can be achieved by people's awareness of the disease and changes in their risk behavior. However, this behavior is difficult to establish, so new or improved methods of surveillance of mosquitoes infected with dengue viruses is necessary to decrease the

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incidence of the disease. Virological surveillance provides an early warning sign for the risk of transmission in an area and for the specific predominant circulating serotype in the vector population (van Benthem *et al*, 2002).

Laboratory tests for the detection of mosquitoes infected with dengue viruses include isolation of the virus and demonstration of a specific viral antigen or RNA. Isolation of the virus is the most definitive approach, but the techniques involved require a relatively high level of technical skill, equipment, and are time-consuming (Rosen and Gubler, 1974), whereas direct detection of dengue antigen, such as dengue antigen-capture ELISA, is not sensitive and has high rates of false positives (Sithiprasasna *et al*, 1994). So, detection of nucleic acid is an alternative method to detect infected mosquitoes.

Polymerase chain reaction (PCR) is one technique available for the laboratory diagnosis of dengue infection. This molecular technique is rapid, highly sensitive and specific. However, PCR must still be viewed as an experimental approach because a consensus has not been reached on the optimal standardization of each step of the procedure, and the requirement of a relatively expensive equipment, a thermalcycler (Lanciotti, 2003).

A method based on nucleic acid sequence-based amplification (NASBA) has been developed to detect dengue viral RNA. NASBA is an isothermal RNA amplification technique that is achieved by the reaction of 3 enzymes; usually avian myeloblastosis-reverse transcriptase (AMV-RT), T7-RNA polymerase, and RNase-H (Compton, 1991). The amplification products can be detected by agarose gel-electrophoresis or electrochemiluminescence (ECL). This method gives 100% sensitivity, 96.30% specificity, and 98.15% efficacy for serum samples. NASBA is, therefore, useful in detecting dengue virus infection (Wu *et al*, 2001; Usawattanakul *et al*, 2002a,b). However, this technique has never been tested with mosquitoes infected with dengue viruses. In this study, NASBA was developed to detect mosquitoes infected with dengue viruses.

MATERIALS AND METHODS

Virus strains

Virus strains in C6/36 cell lines were obtained from the Department of Virology, Armed Forces Research Institute of Medical Sciences (AFRIMS). Dengue virus type 1 (Hawaii), dengue virus type 2 (New Guinea C), dengue virus type 3 (H-87) and dengue virus type 4 (H-241) were titrated in VERO cells by a standard plaque assay.

Mosquitoes

Eggs of *Ae. aegypti* mosquitoes on dry filter paper, received from the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, were reared to adults in the laboratory. Three to five days old adult female *Ae. aegypti* mosquitoes were collected by manual aspirators for use in the experiments and some were kept at -70ºC for use as negative controls in NASBA and PCR detection.

Sensitivity and specificity of dengue virus serotypes

For the specificity test, 100 µl of 10^3 PFU DENV-1, DENV-2, DENV-3, and DENV-4 were inoculated into 5 adult female *Ae. aegypti* mosquitoes. For the sensitivity test, each type of dengue virus was diluted with RPMI media to 10^3, 10^2, 10, 1, and 0.1 PFU, respectively. Each dilution was inoculated into 5 adult female *Ae. aegypti* mosquitoes. After RNA extraction, products were detected by NASBA and PCR.

Amplification of dengue viruses in mosquitoes

Three to five days old adult female *Ae. aegypti* mosquitoes were experimentally infected with 34 PFU (0.34 µl) of DENV-2 using a sterile parenteral inoculation technique (Rosen and Gubler, 1974). Mosquitoes were immobilized over wet ice for 5-10 minutes

before being injected with virus suspension in the membrane area of the intrathoracic. Inoculation procedures took place under a dissecting microscope using a calibrated capillary needle and syringe plunger. Infected mosquitoes were maintained on 10% sucrose at 32ºC for 14 days. They were collected at 0, 7, and 14 days after inoculation and separated into head, thorax, and abdomen. Each part was extracted and subjected to NASBA and PCR.

Selection of primers and probes of NASBA

NASBA is achieved with 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 potato leaf virus sequence that is homologous to the ruthenium-labeled detector probe. The universal capture probe (PR) and 4 serotype-specific capture probes are all in the sense orientation and are immobilized onto the surface of a magnetic bead by means of a streptavidin-biotin linkage. The primers and probes used in this study are listed in Table 1.

RNA extraction

Mosquito samples were squeezed with pullet and mixed with lysis buffer (5.25 mM GuSCN, 50 mM Tris-HCl pH 6.4, 20 mM EDTA, and 1.3% (w/v) TritonX-100). After centrifugation, silica extraction was performed as described by Boom *et al* (1990). In brief, activated silica suspension (50 µl; 1 mg/ml in 0.1 M HCl) was added. After incubation, silica pellet was washed twice with washing buffer (5.25 M GuSCN, 50 mM Tris-HCl, pH 6.4), twice with 70% ethanol and once with acetone. The pellet was dried at 56ºC for 10 minutes. Finally, nucleic acid was eluted with elution buffer (1 mM Tris-HCl pH 8.0, 1 mM EDTA). The mixture was stored at -70ºC until used.

Nucleic acid sequence-based amplification (NASBA)

In brief, the extracted nucleic acid was amplified with 20 µl of reaction mixture (40 mM Tris-HCl, pH 8.5, 12 mM $MgCl_2$, 70 mM KCl, 1.5% (v/v) of dimethyl sulfoxide, 5 mM dithiothreitol, 1mM of each dNTPs, 2 mM of each ATP, CTP, UTP, 1.5 mM GTP, 0.5 mM ITP, 0.2 µM of each primer and 5 µl of isolation nucleic acid) provided by Organon Teknika, Inc, Durham, the Netherlands. The mixture was incubated at 65ºC for 5 minutes, and then cooled down at 41ºC for 5 minutes in a heating box. After addition of enzyme solution (0.1 U RNase-H, 0.1 µg/µl bovine serum albumin, 40 U T7-RNA polymerase, 8 U AMV-reverse transcriptase), the mixture was incubated at 41ºC in the water bath for at least 90 minutes. This product was stored at -20ºC or was used for detection immediately.

Electrochemiluminescence detection

NASBA product was detected by electrochemiluminescence (ECL) method. ECL detection could screen positive pools by using a universal probe and could identify the type of dengue virus by using 4 specific probes (Table 1). Briefly, the NASBA products were diluted to 1:20 in detection diluent (1.0 mM Tris-HCl, pH 8.5, 0.2 g/l methylisothiazolone), incubated with biotinylated dengue virus specific probe bound to 5 µg of streptavidin-coated paramagnetic beads and 3 x 10^{11} molecules of ruthenium-labeled oligonucleotide detection probe. After incubation at 60ºC for 5 minutes and 41ºC for 30 minutes, assay buffer (100 mM tripropylamine, pH 7.5) was added to each tube. The assay result was read by the NASBA QR System, Model 2000 (Organon Teknika, Inc, Durham, the Netherlands).

Polymerase chain reaction (PCR)

Dengue viral RNA in mosquitoes were detected by reverse transcriptase polymerase chain reaction (RT-PCR) using dengue-specific primers (Table 1) and their products were used as the template for nested-PCR reaction using D1, TS1bis, TS2, TS3 and TS4 primers (Table 1), as described by Lanciotti *et al* (1992).

Table 1
Primers and probes for NASBA and PCR.

	Sequence (3′ to 5′)	Location
NASBA		
P1	5′-aat tct aat acg act cac tat agg gga gac	(T7 promotor)
	AGC AGG ATC TCT GGT CT-3′	10,632-10,653
P2	5′-gat gca agg tcg cat atg agg gtt aga gga	(ECL tail)
	GAC CCC TCC C-3′	10,497-10,516
DVP1	5′-GGGAAGCTGTATCCTGGTGGTAAGG-3′	10,550-10,574
DVP2	5′-ATGAAGCTGTAGTCTCACTGGAAGG-3′	10,557-10,581
DVP3	5′-AGGGAAGCTGTACCTCCTTGCAAAG-3′	10,530-10,554
DVP4	5′-GAGGAAGCTGTACTCCTGGTGGAAG-3′	10,483-10,507
PR	5′-AAA CAG CAT ATT GAC GCT GGG-3′	10,615-10,638
PCR		
D1	5′-TCA ATA TGC TGA AAC GCG CGA GAA ACC G-3′	134-161
D2	5′-TTG CAC CAA CAG TCA ATG TCT TCA GGT TC-3′	616-644
TS1bis	5′-CGT CTC AGT GAT CCG GGG RC-3′	568-586
TS2	5′-CGC CAC AAG GGC CAT GAA CAG-3′	232-252
TS3	5′-TAA CAT CAT CAT GAG ACA GAG C-3′	400-421
TS4	5′-CTC TGT TGT CTT AAA CAA GAG A-3′	506-527

In this study, TS1bis (Reynes *et al*, 2003) was used instead of TS1. This primer is degenerated at position 568 (R = A or G) and has one extra nucleotide (C) designed to anneal to position 567. TS1bis allowed amplifying DENV-1 that was not amplifiable by using the original TS1 primer from protocol of Lanciotti *et al* (1992).

Agarose gel-electrophoresis

The RT-PCR product and nested-PCR product were analyzed by agarose gel-electrophoresis on a 1.5% agarose gel (Invitrogen) containing ethidium bromide (0.5 µg/ml). For the DNA size marker, 100 bp DNA ladder (0.1 mg/ml) was used. Electrophoresis was set at 100 volts/cm^2 and was run for 30-45 minutes. The expected size of 482, 119, 290, and 389 bp was identified as being of DENV-1, DENV-2, DENV-3, and DENV-4, respectively.

RESULTS

Specificity of dengue virus serotypes

Extracted dengue viral RNA from mosquitoes infected with 10^3 PFU of each serotype specific dengue virus was detected by NASBA and PCR assays. For PCR, each dengue viral serotype showed a band of amplified DNA fragments at 511 bp in the RT-PCR step, whereas nested-PCR showed clearly distinguished band of 482 bp, 119 bp, 290 bp, and 392 bp for DENV-1, DENV-2, DENV-3, and DENV-4, respectively. For NASBA, all dengue virus serotypes were specifically detected with high signal (data not show).

Sensitivity of dengue virus serotypes

Mosquitoes inoculated with each diluted dengue viral serotype (10^3, 10^2, 10, 1, and 0.1 PFU, respectively) were detected with NASBA and PCR assays. For the NASBA assay, the detection limit of DENV-1 and DENV-3 were 1 PFU, whereas DENV-2 and DENV-4 were below 0.1 PFU (Fig 1). For PCR assay, the detection limit of DENV-1 and DENV-3 were 1 PFU whereas that of DENV-2 and DENV-4 were at 10 PFU (Fig 2).

Detection of dengue virus in infected mosquitoes

Laboratory mosquitoes infected with den-

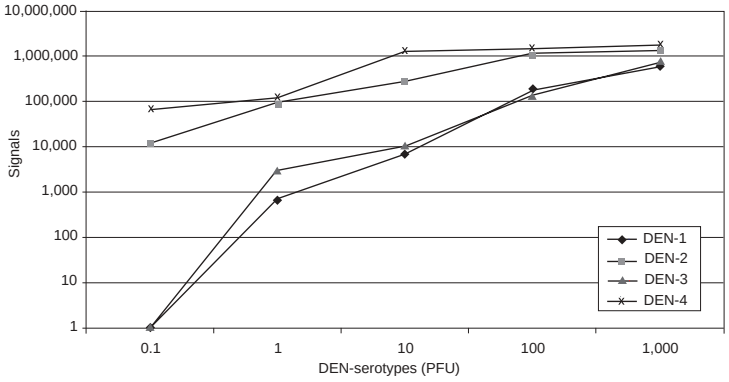

Fig 1–Sensitivity test of NASBA based on ECL analysis for the detection of dengue viral RNA in mosquitoes. Each dilution of 10^3, 10^2, 10, 1, and 0.1 PFU of dengue virus in RPMI media was inoculated into 5 adult female *Ae. aegypti* mosquitoes. After RNA extraction, products were detected by NASBA. Mean values of resulting raw ECL signals are provided on a log scale. The detection limit of DENV-1 and DENV-3 were 1 PFU, whereas DENV-2 and DENV-4 were below 0.1 PFU.

gue viral serotype 2 were dissected into 3 parts (head, thorax, and abdomen) on day 0, 7 and 14 post-inoculation. Each part of the dissected mosquito was amplified by NASBA and PCR to detect dengue virus. For NASBA detection, amplified signals were detected in all parts of the mosquito, with most located in the thorax on day 7 and 14 (Fig 3). The head of an infected mosquito had higher signal than the abdomen.

The results of PCR detection showed the presence of

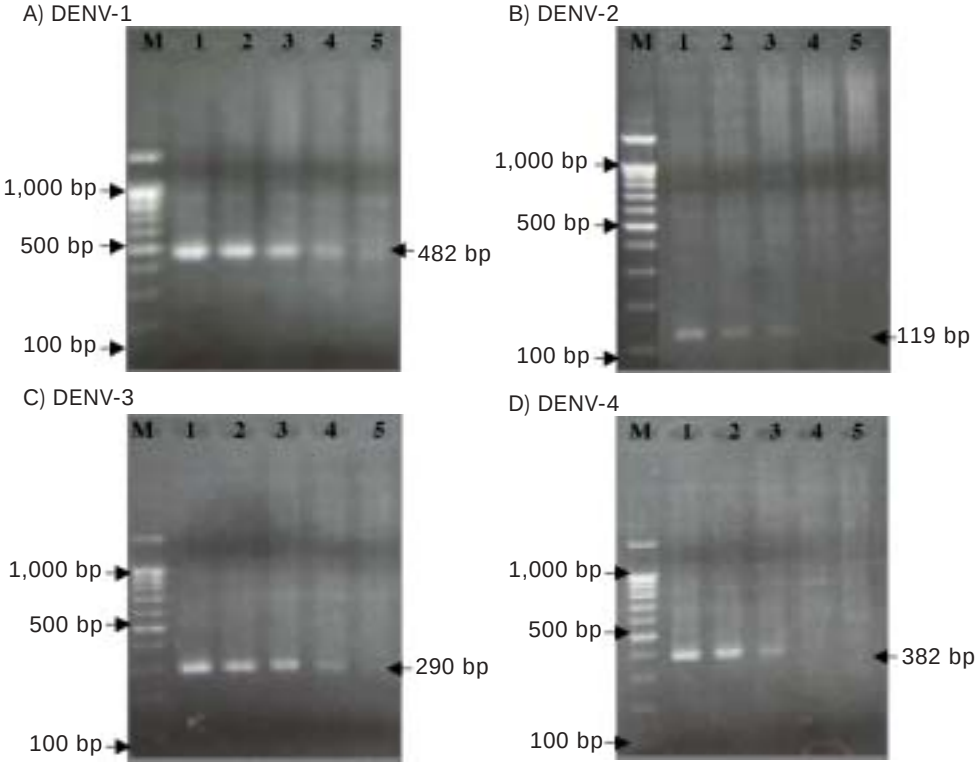

Fig 2–Sensitivity of PCR assay for the detection of dengue viral RNA in the mosquitoes. Each dilution of 10^3, 10^2, 10, 1, and 0.1 PFU of dengue virus in RPMI media was inoculated into 5 adult female *Ae. aegypti* mosquitoes. After RNA extraction, PCR assay was performed and the products were detected by the agarose gel-electrophoresis. Lanes 1-5 was 10^3, 10^2, 10, 1, and 0.1 PFU, respectively. Lane M contains molecular weight markers, DNA sizes are given in base pair.

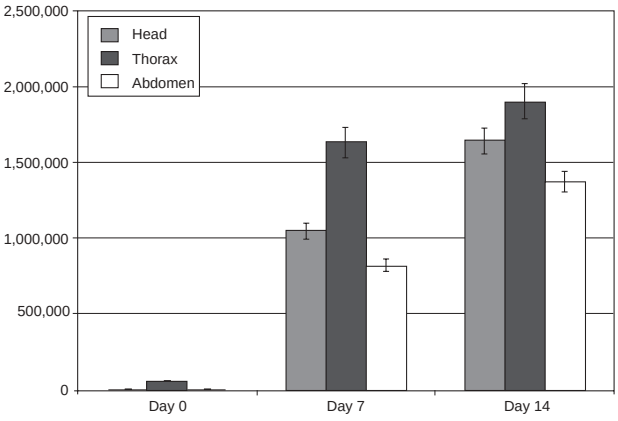

Fig 3–Detection of amplified dengue viruses in mosquitoes by NASBA. Adult female *Ae. aegypti* mosquitoes were experimentally infected with DENV-2 using a parenteral inoculation technique. Infected mosquitoes were maintained on 10% sucrose and collected at day 0, 7, and 14 after inoculation. Each mosquito was separated into head, thorax, and abdomen; then each part was extracted and subjected to the assay. Most of the dengue viruses were found in the thorax.

dengue virus from the first day until the last day of infection. However, the band intensity of PCR product at the first day of inoculation (day 0) and day 7 of post-inoculation were not different in the head, thorax, and abdomen, but the most intense band from the thorax appeared on day 14 post-inoculation.

DISCUSSION

Sensitivity of detection of the four dengue virus serotypes in mosquitoes by NASBA assay was between 0.1 to 1 PFU, which was similar to the detection in serum samples (Wu *et al*, 2001). PCR assay could detect dengue viral RNA of 1 to 10 PFU in mosquitoes. However, it is difficult to compare these results with those of Lanciotti *et al* (1992), because in the later study, the amount was expressed as virus particles with a minimum of 100 complete virus particles being detected.

In inoculated mosquitoes, dengue viruses were detected in the head, thorax, and abdomen of mosquitoes from the first day of inoculation, which may indicate that the virus could spread throughout the mosquito after intrathoracic inoculation. From the results of 7 and 14 day post-inoculation, we showed that most of dengue viruses were present at the salivary gland. The study of Rosen *et al* (1985) showed that all parts of inoculated *Ae. aegypti* have lower titers than orally infected mosquitoes.

An RNA isolation method for the detection of dengue virus in infected mosquitoes requires no RNA degradation. RNases are released during RNA extraction, and their activity must be inhibited as quickly as possible by RNase inhibitors or chaotropic agents (Harris *et al*, 1998; Pankhong *et al*, 2002). In this study, silica combined with the guanidinium thiocyanate-containing lysis buffer was used for extraction because this method can circumvent loss of sensitivity possibly due to unidentified inhibitory components that may be present in biological specimens (Chungue *et al*, 1993) and lysis buffer reproducibly yielded the highest quality RNA because of the extremely chaotropic nature of this chemical (Boom *et al*, 1990).

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used in this study was modified from the original method of Lanciotti *et al* (1992), which showed that no cross reaction between dengue virus and five dengue virus-related flaviviruses (West Nile, Japanese encephalitis, St Louis encephalitis, yellow fever, and Edge Hill), when using dengue virus type-specific oligonucleotide primers. Initially, TS1 primer of Lanciotti *et al* (1992) could not identify dengue virus serotype 1. The amplification failure of nested-PCR might be related to the presence of a DENV-1 variant, resulting in a primer-template mismatch. Therefore, a new primer, TS1bis, was used to improve this method. Reynes *et al* (2003) reported that PCR

sensitivity with TS1bis is equivalent to that with TS1 and this specific probe does not alter the specificity of the technique, since genomes of DENV-2, DENV-3, DENV-4, and other related flaviviruses such as yellow fever, West Nile, Japanese encephalitis are not amplified.

From our results, we can not conclude that PCR has low sensitivity because dengue virus consensus primers (D1 and D2) and serotype specific primers were designed from available published sequences with the aid of a sequence analysis computer program (Lanciotti *et al*, 1992), and these primers may have not annealed specifically enough with dengue virus genomes present in Thailand.

The NASBA assay has several advantages over the PCR method. The amplification process of the NASBA uses an isothermal temperature (41ºC), so that a thermalcycler is not required. Moreover, the relatively low temperature of NASBA process allows for the annealing of primers with less than 100% homology. The NASBA product is single stranded RNA and can therefore be readily detected by hybridization analysis without the denaturation steps and contamination with genomic DNA is not amplified.

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