MOLECULAR SEROTYPING OF DENGUE VIRUSES IN FIELD-CAUGHT *AEDES* MOSQUITOS BY IN-HOUSE RNA EXTRACTION/ RT-PCR REAGENT KITS

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Abstract. We developed in-house RNA extraction and RT-PCR reagent kits for the molecular serotyping of dengue viruses in field-caught *Aedes* mosquitos. Mosquitos that showed positive results by ELISA or IFA were selected for the identification of dengue viruses in order to predict the distribution of the four dengue serotypes. Total RNA was extracted from one whole mosquito as well as from one dissected mosquito by our in-house RNA extraction reagents using the modified method of guanidinium thiocyanate denaturation and isopropanol precipitation. The extracted RNA was amplified by our in-house RT-PCR reagents specific for each dengue serotype under optimized conditions. Dengue viral RNA extracted from a single mosquito as well as from the head and thorax of one dissected mosquito could be detected successfully; it could not be found in the abdomen, however. These results indicated that most of the dengue viruses were located in the head and thorax rather than in the abdomen. The results of dengue serotyping showed a pure specific PCR product for each dengue serotype at 490, 230, 320 and 398bp for DEN-1, DEN-2, DEN-3, and DEN-4 respectively. In addition, the detection sensitivity was very high: an amount of RNA template equivalent to approximately 1/80 of a single mosquito could be detected by agarose gel electrophoresis and ethidium bromide staining. The coupling of RT-PCR- based surveillance of dengue viral infection with disease mapping data (Geograpical Information System, GIS) could serve as an alternative epidemiological means of providing an early warning of dengue fever/ dengue hemorrhagic fever epidemics.

INTRODUCTION

The genus *Flavivirus* of the family Flaviviridae includes viruses that cause diseases of major health importance, such as yellow fever (YF), Japanese encephalitis (JE), tick-borne encephalitis (TBE), and dengue (DEN). There are four antigenically related, but distinct, dengue virus serotypes (DEN-1, DEN-2, DEN-3, DEN-4), all of which can cause a range of diseases: dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). DEN diseases cause death and hospitalization in tropical and subtropical countries around the world. Dengue viruses are enveloped RNA viruses that contain a single-stranded, positive-sense RNA of approximately 11kb in length. The genomic RNA has type-I 5´ cap of m^7Gppp^A and lacks a 3´ -end poly(A) track (Wengler and Wengler, 1981; Brinton *et al*, 1986; Brinton and Disposito, 1988). The flavivirus genome encodes an uninterrupted open reading frame (ORF), flanked by 5´ and 3´ non-coding regions. The order of proteins encoded in dengue viruses ORF is: 5´-non coding region (5´-NC)-capsid(C)-premembrane/membrane (prM/M)-envelope (E)-nonstructural proteins -NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5- 3´-non coding region (3´- NC) (Westaway *et al*, 1985). Dengue viruses are transmitted to human by domestic *Aedes* mosquitos, which inhabit the tropics and subtropics, raising the spectre of endemic dengue in these areas. The previous data suggested that dengue viruses were isolated from *Aedes* mosquitos collected in nature (Khin and Than, 1983; Hull *et al*, 1984). Therefore, the identification and typing of dengue viruses isolated from the field-caught *Aedes* mosquitos and from clinical specimens are important for epidemiological and clinical investigations. The technique that is simple and rapid for the detection, identification, and typing of dengue viruses is reverse transcription - polymerase chain reaction (RT-PCR). We present our development of an in-house RNA extraction and RT-PCR reagent kits for the molecular serotyping of dengue viruses in field-caught *Aedes* mosquitos.

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MATERIALS AND METHODS

Mosquitos

Two types of mosquitos, including laboratory-infected mosquitos and field-caught mosquitos, were used in our study. The laboratory infected mosquitos were female adult *Aedes* mosquitos infected with DEN-1, DEN-2, DEN-3, DEN-4 that were provided by the Department of Entomology and Virology, the Armed Forces Research Institute of Medicial Sciences (AFRIMS), Thailand. The field-caught mosquitos were female adult *Aedes* which were caught in selected dengue-sensitive areas in Chom Bung district, Ratchaburi Province, Thailand. Each field-caught mosquito was dissected into three parts for three different analyses: the head was tested by IFA; the thorax was tested by ELISA; the remaining part of head and the abdomen were analyzed by RT-PCR.

RNA extraction

The total genomic RNA of the laboratory-infected mosquitos and the field-caught mosquitos was extracted by the modified method of guanidinium thiocyanate denaturation and isopropanol precipitation. The starting material was the single-ground mosquito digested in 100µl of the lysis buffer, which contained 6M guanidine thiocyanate and 10µl of 10mg/ml Proteinase K. The mixture was incubated at 55°C for 1 hour. Then, the mixture was centrifuged at 10,000rpm for 10 minutes and the aqueous phase was transferred to a new microtube. The aqueous phase was mixed with an equal volume of isopropanol and incubated at -20°C for at least 30 minutes. After being centrifuged at 4°C, the supernatant was removed. The pellet was washed with 70% (v/v) ethanol and air-dried. Finally, the total genomic RNA was dissolved in 40µl of RNase-free TE buffer in the presence of the RNasin ribonuclease inhibitor (Promega, USA) and stored at -70°C until use.

Reverse transcription and PCR amplification

Each reaction mixture contained the following components: 1 × PCR buffer (100mM Tris-HCl pH8.3, 500mM KCl, 25mM $MgCl_2$, 1 mg/ml gelatin, 0.2% v/v NP-40), 2mM dNTPs, 7pmol/µl of each serotype-specific primer (Morita *et al*, 1991), 1U of Taq DNA polymerase (Pharmacia Biotech, USA), 2U of AMV-RT (Promega, USA) and 5µl of the extracted RNA was used as template in a 25µl reaction volume. The reaction mixture was covered with 30µl of mineral oil. Reverse transcription was conducted at 53°C for 20 minutes, followed by 35 amplification cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes. Amplification was conducted in 0.6ml microtube with a Hybaid Omni Gene (Hybaid, England). Finally, all of the products were stored at -20°C until use.

Analysis of PCR products

Each PCR product (5-10µl) was analyzed by standard horizontal gel electrophoresis using 2% SeaKem LE agarose (FMC, USA) in Tris-borate/EDTA buffer, visualized by ethidium bromide staining, and videographed under UV transillumination using a Video CCD Camera (Vilber Lourmat, France).

RESULTS

Specificity of RT-PCR detection and serotyping of dengue virus in laboratory-infected mosquitos

Dengue viral RNA extracted from each laboratory-infected mosquito could be serotyped successfully by our in-house RNA extraction/ RT-PCR reagents. Each dengue serotype showed a single band of amplified DNA fragments at 490, 230, 320 and 398bp for DEN-1, DEN-2, DEN-3 and DEN-4 respectively (Fig 1).

Extracted RNA from each uninfected mosquito was examined. All of the extracted RNA samples were amplified by RT-PCR using a specific primer pair for each dengue serotype. The results of this examination of uninfected mosquitos showed no amplified DNA fragments (Fig 2).

Detection sensitivity

Varying amounts of the extracted RNA (5, 2.5, 1,

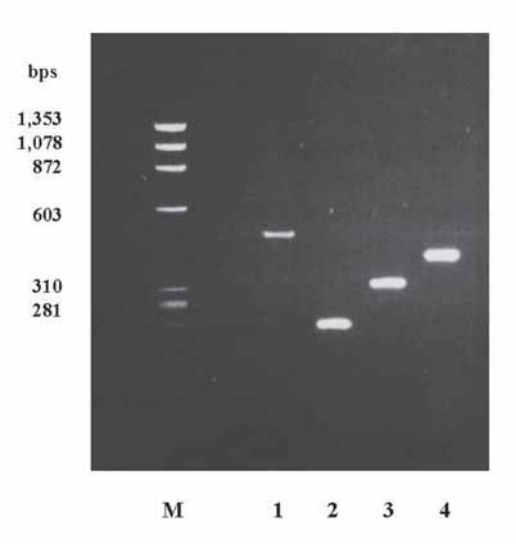

Fig 1- The amplified fragment of each dengue serotype. M is the standard DNA marker, lanes 1, 2, 3, and 4 are four single bands of amplified DNA fragments at 490, 230, 320 and 398 bp for DEN-1, DEN-2, DEN-3, and DEN-4 respectively.

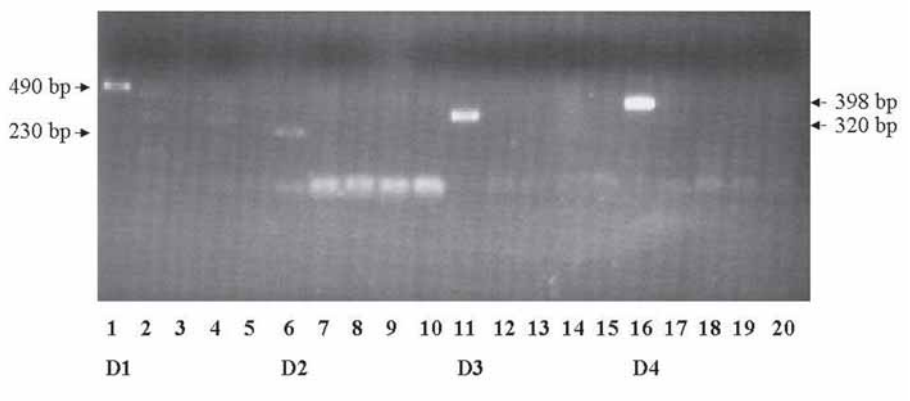

Fig 2- Specificity of four pairs of dengue serotype specific primers. Four RNA samples of uninfected mosquitos were analyzed by RT-PCR using a primer pair specific for each dengue serotype. The PCR products were analyzed by agarose gel electrophoresis stained with ethidium bromide : lanes 1-5 for DEN-1; lanes 6-10 for DEN-2; lanes 11-15 for DEN-3; lanes 16-20 for DEN-4. D1, D2, D3, D4 are specific DNA markers for each dengue serotype (lanes 1, 6, 11, and 16 respectively).

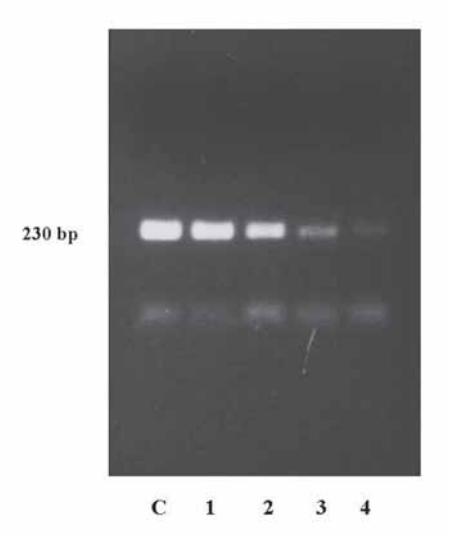

Fig 3- Detection sensitivity of DEN-2 in a single infected *Aedes* mosquito by RT-PCR and agarose gel electrophoresis stained with ethidium bromide. C is the positive control, lanes 1, 2, 3, and 4 are various amounts of RNA template at 5, 2.5, 1, 0.5µl, respectively.

0.5µl) from each laboratory mosquito infected with DEN-2 were amplified by RT-PCR using primers D2S and D2C (Morita *et al*, 1991) to determine the detection limit. After 35 cycles, the target PCR product amplified from 0.5µl of each RNA sample, or approximately 1 in 80 parts of one mosquito, could be visualized (Fig 3). In addition, the amplified dengue cDNA could be detected from head and thorax of one dissected mosquito but not from the abdomen (Fig 4). These results indicated that most of the dengue viruses were located in head and thorax rather than the abdomen.

Detection of dengue virus in the field-caught *Aedes* mosquitos

The remaining sample of each dissected field-caught mosquito that showed positive results by either IFA or ELISA was further analyzed by RT-PCR. The remaining dissected mosquitos were divided into two groups: the first group (no.1-5) comprised mosquitos that gave positive results by IFA and ELISA that were higher than those of the second group (no.6-20). The first RT-PCR analysis was conducted using primers D2S and D2C because DEN-2 were always predominately epidemic in Thailand. Among the 20 field-caught mosquitos of both groups, the results indicated that only three positive mosquitos were infected with DEN-2 (Fig 5). However, the remaining samples that were negative for DEN-2 were further analyzed by RT-PCR using DEN-3 specific primers (D3S/D3C): no amplified specific product was detected in any of them (data not shown).

DISCUSSION

Dengue virus classification into four serotypes is traditionally based on antigenic characteristics determined by plaque neutralization (Russell and Nisalak, 1967; Calisher *et al*, 1989), by immunofluorescence in cell culture (Henchal *et al*, 1983) and by complement fixation (Kuberski and Rosen, 1977; Tesh, 1979). All these techniques can not specifically identify dengue serotypes in clinical specimens and field-caught mosquitos. Amplification

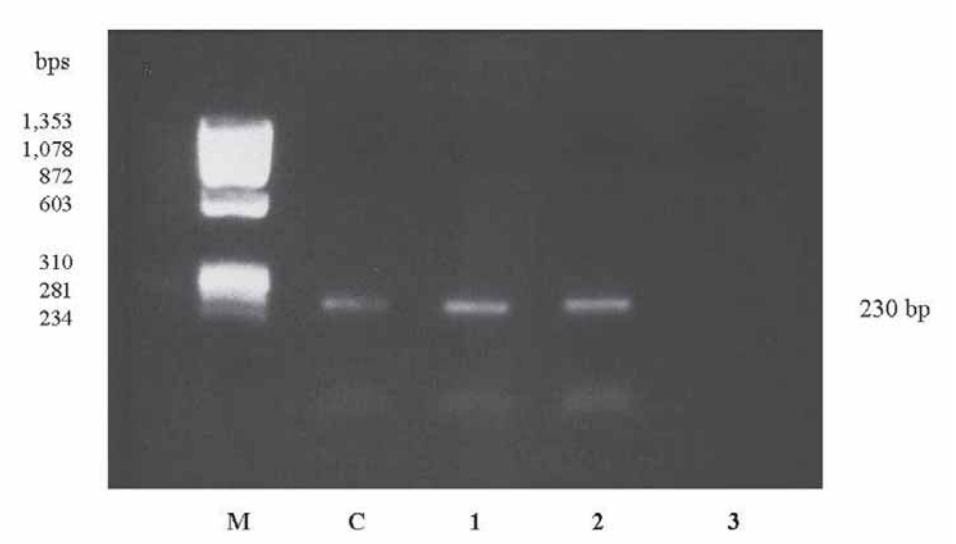

Fig 4- The PCR products of the RNA extracted from three parts of a single dissected mosquito. A single mosquito infected with DEN-2 was dissected into 3 parts: head, thorax, and abdomen. The RNA extracted from each part was analyzed by RT-PCR and agarose gel electrophoresis followed by ethidium bromide staining. M is the standard DNA marker; C is the positive control. Lanes 1-3 are the PCR products of the RNA extracted from the head (lane 1), thorax (lane 2) and abdomen (lane 3).

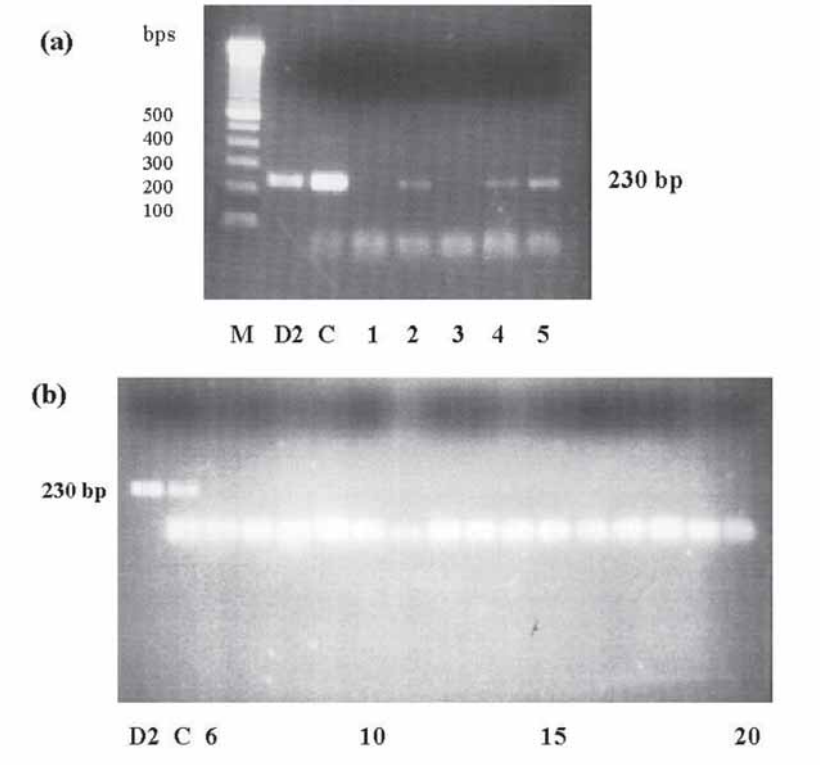

Fig 5- Detection of dengue virus in twenty field-caught *Aedes* mosquitos by RT-PCR and agarose gel electrophoresis followed by ethidium bromide staining. (a) The first group of five mosquitos that gave high positive results by IFA and ELISA. (b) The second group of fifteen mosquitos that gave low positive results by IFA and ELISA. M is the standard DNA marker; D2 is the specific DNA marker for DEN-2; C is the positive control. Lanes 1-20 are the PCR products of RNA extracted from twenty field-caught mosquitos.

of the conserved dengue RNA target by RT-PCR is an appropriate technique for dengue serotyping directly from such samples. In-house RNA extraction and RT-PCR reagent kits have been presented in this paper and have been validated for the molecular serotyping of dengue viruses in field-caught *Aedes*. The sensitivity and specificity of dengue viruses serotyping were emphasized in our validation.

The amount of dengue viral RNA that can be obtained from each field-caught mosquito varies considerably, depending on the virus titer, the preservation of samples, and the method of RNA extraction. The isolation of dengue viral RNA from a veriety of sources requires the taking of precautions in order to avoid RNases. RNases are released during RNA extraction, and their activity must be inhibited as quickly as possible by RNases inhibitors or chaotropic agents which denature proteins. Chomezynski and Sacchi (1987) described the isolation and purification of undegraded RNA by a guanidinium thiocyanate-containing lysis buffer. This lysis buffer reproducibly yielded the highest quality RNA because of the extremely chaotropic nature of this chemical: it is one of the most effective protein denaturants (Boom *et al*, 1990). The RNA extraction protocol that includes our in-house reagents is the modified guanidinium thiocyanate method that was described by Attatippaholkun *et al* (1998). Our simplified protocol allowed the rapid isolation of dengue viral RNA from each field-caught *Aedes* mosquito. In addition, the extracted dengue RNA remained preserved in the presence of an RNasin ribonuclease inhibitor (Promega, USA) at -70˚C until use. Following the RT-PCR method of dengue serotyping established by Morita *et al* (1991), our in-house reagents, as well as the four pairs of serotype specific primers, were locally prepared for serotyping dengue viruses in each field-caught *Aedes*. Using experimental *Aedes* that were either uninfected or infected with various dengue serotypes, our in-house RNA extraction and RT-PCR reagents showed high specificity and sensitivity (Figs 1 and 2). Moreover, dengue viral RNA could be detected in a single mosquito, as reported by Eva *et al* (1998). The detection sensitivity required approximately 1 in 80 parts of the total RNA extracted from a single mosquito (Fig 3). For localization of dengue viruses in *Aedes*, our data indicated that most of dengue viruses were located in the head and thorax, rather than the abdomen (Fig 4).

It has been reported previously that the abdomen of the *Aedes* contains the least dengue virus in comparison with the head and thorax. The RT-PCR results of twenty field-caught mosquitos, positive by IFA and ELISA, showed that only three of them were infected with DEN-2 (Fig 5). One possible reason for these RT-PCR results was the fact that these mosquitos, analysed after IFA and ELISA, were not treated correctly because no precautions had been taken regarding RNase inhibition. On the other hand, the mosquitos that gave negative results for DEN-2 and DEN-3 might have been infected by the other dengue serotypes: we did not have enough RNA templates for further analyses.

However, our established RT-PCR including the in-house RNA extraction and the RT-PCR reagents could be applied for dengue serotyping in field - caught *Aedes* mosquitos. The method presented in this paper could make for the rapid and specific identification of dengue viruses in a single *Aedes* mosquito - useful for surveillance and the epidemiological study of these viruses. The geographic distribution of virus-infected *Aedes* and household locations has also been managed by using the Global Positioning System (GPS) and the Geographical Information System (GIS). Data from RT-PCR-based surveillance of dengue coupled with disease mapping data could serve as a useful epidemiological tool that might provide early warnings of dengue fever/ dengue hemorrhagic fever epidemics.

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REFERENCES

Attatippaholkun WH, Attatippaholkun MK, Nisalak A, Vaughn DW, Innis BL. Nucleotide sequence and deduced amino acid type 3 virus, Bangkok genotype. *Southeast Asian J Trop Med Public Health* 1998;29:540-6.

Boom R, Sol CJA, Salimans MMM, *et al*. Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* 1990;28:495-503.

Brinton MA, Disposito JH. Sequenced and secondary structure analysis of the 5´- terminal region of flavivirus genome RNA. *Virology* 1988;162:290-9.

Brinton MA, Fernandez AV, Amato J. The 3´-nucleotides of flavivirus genome RNA form a

conserved secondary structure. *Virology* 1986; 153: 113-21.

Calisher CH, Karabatsos N, Dalrymple JM, *et al*. Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. *J Gen Virol* 1989;70:37-43.

Chomezynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analyt Biochem* 1987;162: 156-9.

Eva H, T Guy R, Leila S, *et al.* Typing of dengue viruses in clinical specimens and mosquitoes by single-tube multiplex reverse transcription PCR. *J Clin Microbiol* 1998;36:2634-9.

Henchal EA, McCown JM, Seguin MC, Gentry MK, Brandt WE. Rapid identification of dengue virus isolates by using monoclonal antibodies in an indirect immunofluorescence assay. *Am J Trop Med Hyg* 1983;32:164-9.

Hull B, Tikasingh E, De Souza M, Martinez R. Natural transovarial transmission of dengue 4 virus in *Aedes aegypti* in Trinidad. *Am J Trop Med Hyg* 1984;33:1248-50.

Khin MM, Than KA. Transovarial transmission of dengue 2 virus by *Aedes aegypti* in nature. *Am J Trop Med Hyg* 1983;32:590-4.

Kuberski TT, Rosen L. Identification of dengue viruses using complement fixing antigen produced in mosquitoes. *Am J Trop Med Hyg* 1977;26:538-43.

Morita K, Tanaka M, Igarashi A. Rapid identification of dengue virus serotypes by using polymerase chain reaction. *J Clin Microbiol* 1991;29:2107-10.

Russell PK, Nisalak A. Dengue virus identification by the plaque reduction neutralization test. *J Immunol* 1967;99:291-6.

Tesh RB. A method for the isolation and identification of dengue viruses using mosquito cell cultures. *Am J Trop Med Hyg* 1979;28:1053-9.

Wengler G, Wengler G. Terminal sequences of the genome and replication form RNA of the flavivirus West Nile virus; absence of poly(A) and possible role in RNA replication. *Virology* 1981;113:544-94.

Westaway EG, Brinton MA, Gaidamovich SY, *et al*. Flaviviridae. *Intervirology* 1985;24:183-92.