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Original Article

Efficiency comparison of four high-fidelity DNA polymerases for dengue virus detection and genotype identification in field-caught mosquitoes

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ABSTRACT

Dengue disease is an important arboviral disease caused by the bite of a dengue virus (DENV)-infected mosquito vector, especially *Aedes aegypti*. This disease is widely spread throughout both the tropical and temperate zones. DENV causes deaths every year, especially in children, thus emphasizing the need to improve DENV surveillance. Early detection and accurate serotype and genotype identification is one approach for improving DENV surveillance; therefore, this study evaluated the efficiency of four high-fidelity DNA polymerases—AccuPrime™ *Taq*, Platinum® Pfx, Q5® High-Fidelity, and KOD FX Neo—in amplifying the C/prM junction and the *NS5* and *E* genes that have been widely used to detect DENV and identify a DENV serotype and genotype using a method based on reverse transcription polymerase chain reaction. By amplifying the C/prM junction from DENV isolated from the viral culture, Q5 was selected for screening DENV infection in field-caught mosquitoes. The results of screening 2791 female mosquitoes collected from 2011 to 2015 showed that all DENV serotypes circulated in Thailand with the highest frequency serotype being DENV-3. Then, cDNAs of four pooled mosquitoes detected to carry four different serotypes were selected to examine the efficiency of the DNA polymerases. The results showed that Pfx had the highest efficiency for amplifying the C/prM junction and the partial *NS5* gene, while AccuPrime was the most efficient enzyme for amplifying the complete *E* gene. Hence, these results suggested that both the type of sample and the region of the DENV genome should be considered when choosing an efficient DNA polymerase.

Introduction

Dengue fever, dengue hemorrhagic fever and dengue shock syndrome are severe arboviral diseases caused by the dengue virus (DENV) that are transmitted to human by *Aedes* mosquito vectors, especially *Aedes aegypti* (World Health Organization, 2016). Approximately 3900 million people in 128 countries worldwide are at risk of infection with dengue virus (World Health Organization, 2016). Childhood has a significant risk of dengue diseases; approximately 500,000 children are infected annually with DENV and about 2.5% of them die (World Health Organization, 2016). The number of DENV-infected persons in Thailand has increased from 116,947 cases in 2010 to 144,952 cases in 2015 (Ketkaew et al., 2016). This large and increasing number of dengue cases in Thailand emphasizes the need to improve DENV surveillance.

Dengue virus, belonging to the genus *Flavivirus* within the *Flaviviridae* family, exists as four different serotypes: DENV-1, DENV-2, DENV-3 and DENV-4 (Idrees and Ashfaq, 2012). Correlation between the DENV serotype and disease severity has been observed in several studies (Nisalak et al., 2003; Klungthong et al., 2004; Fried et al., 2010; Yung et al., 2015). Some studies showed that DENV-2 was significantly correlated with severe dengue diseases (Kumaria, 2010; Vicente et al., 2016). Each serotype can be classified into several genotypes which mostly correspond with geographical distribution (Rico-Hesse, 2003). Some genotypes were reported to be related to the virulence of dengue diseases; for example, the DENV-2 Southeast Asia genotype has been observed

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to be associated with the first DHF outbreak in the Americas, while the co-circulating DENV-2 American genotype almost exclusively causes DF (Añez et al., 2011; Drumond et al., 2013). These studies suggested that both DENV serotypes and genotypes are associated with the severity of dengue diseases.

The early detection of DENV infection in humans would be very useful for dengue disease treatment and a capability of detecting DENV infection in mosquito vectors would aid in preventing dengue disease outbreak. There are many methods for detecting DENV including virus isolation, viral genome detection and serological detection (Peeling et al., 2010). Reverse transcription polymerase chain reaction (RT-PCR) is one of the highly sensitive and specific methods which has been widely applied for DENV detection and DENV serotype/genotype identification (Lanciotti et al., 1992; Peeling et al., 2010). The success of using this method for DENV detection depends on many factors including the copy number of the DENV genome in the samples and the sensitivity of detection assay (Lanciotti et al., 1992). In the polymerase chain reaction (PCR) step, DNA polymerase and buffer conditions are important factors directly affecting amplification efficiency (Arezi et al., 2003). Regarding the efficiency of DNA polymerases, Purzycka and colleagues reported that *Taq* DNA polymerase (Invitrogen™, Waltham, MA, USA) has the highest efficiency for amplifying STR loci in human blood samples (Purzycka et al., 2006). The comparison of six commercially-available DNA polymerases that could be applied to detect *Plasmodium* infection using a direct PCR method showed that KOD FX could yield a relatively high amount of the PCR product amplified directly from blood samples containing PCR inhibitors and a mild detergent (Miura et al., 2013). Regarding DENV detection, all previous studies attempted to evaluate the efficiency of either the DENV detection methods or commercial kits for DENV detection; no studies attempted to evaluate the efficiency of DNA polymerase used in the method based on RT-PCR for DENV detection (Ahmed and Broor, 2014; Najioullah et al., 2014; Teoh et al., 2015).

DENV serotype and genotype identification are important because the severity of dengue diseases depends also on the DENV serotype and genotype (Yung et al., 2015). In addition, the DENV genotype determines the transmission potential of dengue virus (Rico-Hesse, 2010; Lequime et al., 2016). Regarding the method based on RT-PCR, the C/prM junction has been used frequently for identifying the DENV serotype because its sequence is highly conserved within the same serotype (Lanciotti et al., 1992; Khawsak et al., 2003). The phylogenetic analysis performed on 11 regions of DENV genomes of every serotype with diverse genotypes showed that each DENV serotype had a different set of genes that were suitable for genotyping (Klungthong et al., 2008); however, the current study supported the use of *NS5* and *E* genes for DENV genotype identification as reported by many previous studies (Domingo et al., 2011; Fatima et al., 2011; Alfonso et al., 2012). Hence, these studies supported the use of the C/prM junction and the partial *NS5* and the complete *E* gene sequences for DENV detection, serotype identification and genotyping.

The current study examined the efficiency of four high-fidelity DNA polymerases—AccuPrime™ *Taq* DNA Polymerase, Platinum® Pfx DNA Polymerase, Q5® High-Fidelity DNA Polymerase and KOD FX Neo—for developing an efficient method based on RT-PCR for detecting and identifying DENV in field-caught mosquitoes. This type of sample was chosen because it normally harbors a very low viral load, which is suitable for developing a highly sensitive method for DENV detection. Three positions of every serotype of DENV genome were chosen—the C/prM junction and the *NS5* and *E* genes. The efficiency of DNA polymerases was evaluated via agarose gel electrophoresis and statistical analysis carried out on the concentration of the PCR products. The results of this study would be useful for developing an efficient method based on RT-PCR for detecting and identifying DENV serotypes and genotypes in any sample carrying a very low viral load.

Materials and methods

Sample preparation and RNA extraction

The viral cell culture and the field-caught mosquito vector were chosen for examining the efficiency of commercially available DNA polymerases. The viral cell culture samples were obtained from the Medical Biotechnology Unit, Siriraj Hospital (Bangkok, Thailand). The dengue virus isolated from the viral culture was 10-fold serially diluted generating 1, 0.1, 0.01 and 0.001 plaque forming units (PFU)/mL for each serotype. RNA of each dengue virus sample was extracted from 140 μL of each diluted sample using a QIAamp Viral RNA Mini Kit (Qiagen; Hilden, Germany) following the manufacturer's protocol.

From 2011 to 2015, *Aedes* mosquito samples were caught using hand nets inside houses located in epidemic areas of 10 provinces in Thailand (Bangkok, Pathum Thani, Nonthaburi, LopBuri, Suphanburi, Chanthaburi, Chasengsao, Trat, Nakon Ratchasima and Songkhla). The species and sex of the *Aedes* mosquitoes were identified using morphological characters (Huang and Rueda, 2014). One to ten female *Aedes aegypti* mosquitoes were pooled by collection site and date. The mosquitoes were kept at −80 °C until used. Wings and legs of each mosquito were removed before RNA extraction. Total RNA of each pooled field-caught mosquitoes was extracted using an RNeasy Mini Kit (Qiagen; Hilden, Germany) according to the manufacturer's protocol.

Reverse transcription polymerase chain reaction (RT-PCR)

RNA samples from viral culture and field-caught mosquito samples were used as templates to synthesize first-strand cDNA using SuperScript®III First-Strand Synthesis System (Invitrogen™; Hercules, CA, USA) and dengue-specific primers (Lanciotti et al., 1992). The procedure of cDNA synthesis followed the manufacture's protocol.

Four commercial DNA polymerase—AccuPrime™ *Taq* DNA Polymerase High Fidelity (Invitrogen™; Hercules, CA, USA), KOD FX Neo (Toyobo; Osaka, Japan), Platinum® Pfx DNA Polymerase (Invitrogen™; Hercules, CA, USA) and Q5® High-Fidelity DNA Polymerase (New England Biolabs; Ipswich, MA, USA)—were first applied to amplify the C/prM junction from the DENV isolated from the viral cell culture. The final concentrations, PCR mixture and PCR condition used in this study are presented in Table 1. According to the methods based on RT-PCR proposed by Lanciotti et al. (1992), D1 and D2 primers were applied to confirm the presence of DENV in the cDNA of the viral cell culture, then the PCR products obtained from this step were used as templates for serotype identification by serotype-specific primers (TS1, TS2, TS3 and TS4) using the semi-nested PCR method (Lanciotti et al., 1992).

These four DNA polymerases were applied to amplify three DENV positions (the C/prM junction and the *NS5* and *E* genes) in the field-caught mosquito samples. Approximately 500 bp of the C/prM junction was amplified using the same primers, PCR condition and duration of extension time as mentioned above. The partial *NS5* gene (approximately 1000 bp) was amplified using a primer pair named FU1−F and cFD3-R (Kuno et al., 1998). The complete *E* gene (approximately 1700 bp) was amplified using serotype-specific primers that were composed of four forward primers—GENE-SS (DENV1), EGENE2-SS (DENV2), EGENE3-SS (DENV3), EGENE4-SS (DENV4)—and one reverse primer named EGENE/NS1-RR (Domingo et al., 2006). The PCR mixture and PCR condition used in this study are shown in Table 1.

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