



## 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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## ARTICLE INFO

## Article history:

Received 29 December 2016

Accepted 22 May 2017

Available online 5 June 2018

## Keywords:

Dengue virus

*Aedes aegypti*

Detection

Genotype

Reverse transcription polymerase chain reaction

## 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.

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## 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; Najjioullah 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  $-80^{\circ}\text{C}$  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 manufacturer'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.

**Table 1**  
Final concentrations of polymerase chain reaction (PCR) components, PCR condition and extension time used for amplifying C/prM junction, partial NS5 gene and complete E gene using four DNA polymerases.

Component	Final concentration of PCR components			
	AccuPrime™	Platinum® Pfx	Q5®	KOD FX Neo
PCR buffer	2X	1X	1X	1X
PCR enhancer	–	1X	1X	–
MgSO <sub>4</sub>	2 mM <sup>a</sup>	1 mM	1 mM <sup>a</sup>	1 mM <sup>a</sup>
dNTP	0.4 mM <sup>a</sup>	0.3 mM	0.2 mM	0.4 mM
Forward primer	0.2 μM	0.3 μM	0.5 μM	0.3 μM
Reverse primer	0.2 μM	0.3 μM	0.5 μM	0.3 μM
DNA polymerase	2.0 units	1 unit	0.02 unit	1 unit
cDNA	4 μL	4 μL	4 μL	4 μL
Nuclease-Free water	to 50 μL	to 50 μL	to 50 μL	to 50 μL
Step	PCR condition			
	AccuPrime™	Platinum® Pfx	Q5®	KOD FX Neo
Initial denaturation	94 °C, 15 s	94 °C, 5 min	98 °C, 30 s	94 °C, 2 min
Denaturation	94 °C, 15 s	94 °C, 15 s	98 °C, 10 s	98 °C, 10 s
Annealing	55 °C, 30 s	55 °C, 30 s	55 °C, 30 s	55 °C, 30 s
Extension <sup>b</sup>	68 °C, A	68 °C, B	72 °C, C	68 °C, D
Final extension	68 °C, 10 min	68 °C, 10 min	72 °C, 2 min	68 °C, 7 min
Position (Size of amplicon)	Time used in the extension step			
	AccuPrime™	Platinum® Pfx	Q5®	KOD FX Neo
C/prM (500 bp)	30 s	30 s	20 s	20 s
E (1700 bp)	2 min	2 min	1.30 min	1 min
NS5 (1000 bp)	1 min	1 min	40 s	30 s

<sup>a</sup> Component already mixed in the PCR buffer.

<sup>b</sup> Times used in the extension step for each region amplified by different enzymes.

#### Screening for presence of dengue virus in field-caught mosquitoes

The C/prM junction was amplified from the cDNA of each set of pooled field-caught mosquitoes using the procedures described above. The DNA polymerase that had the highest sensitivity for detecting DENV generated from the viral cell culture was used in this experiment. The cDNAs of the field-caught mosquito samples observed to carry different DENV serotypes were applied to evaluate the DNA polymerase performance using the method based on RT-PCR described above.

#### Evaluation of DNA polymerase performance

All RT-PCR products were detected using gel electrophoresis in 1.5% of agarose gel (Vivantis; Selangor Darul Ehsan, Malaysia). The DNA Ladder (100bp–10 kb) (PCR Biosystems; London, UK) was used to estimate the size of PCR products. The gel was stained using 0.5 μg/mL of ethidium bromide solution for 30 min. PCR products were viewed using the Molecular Imager® Gel Doc™ XR<sup>+</sup> System with Image Lab™ Software (Bio-Rad Laboratories; Hercules, CA, USA). The size and intensity of the DNA bands representing PCR products were measured by comparison with the DNA ladder and converted to the DNA concentration using quantity tools of the Image Lab™ Software. If non-specific PCR products were produced, only the size and intensity of the positive DNA band were measured and converted to the PCR product concentration.

The efficiency of each DNA polymerase was assessed based on the lowest concentration of the DNA template that could be amplified by the enzyme (sensitivity of the enzyme), the genuineness of the PCR product (specificity of the enzyme), and the concentration of the PCR product (productivity of the enzyme). In order to test the productivity of different enzymes applied to amplify a particular region of the DENV genome from different DENV serotypes, a two-way analysis of variance (ANOVA) and its following pairwise comparisons (Tukey's HSD test) were applied to compare the PCR product concentrations. Both the two-way

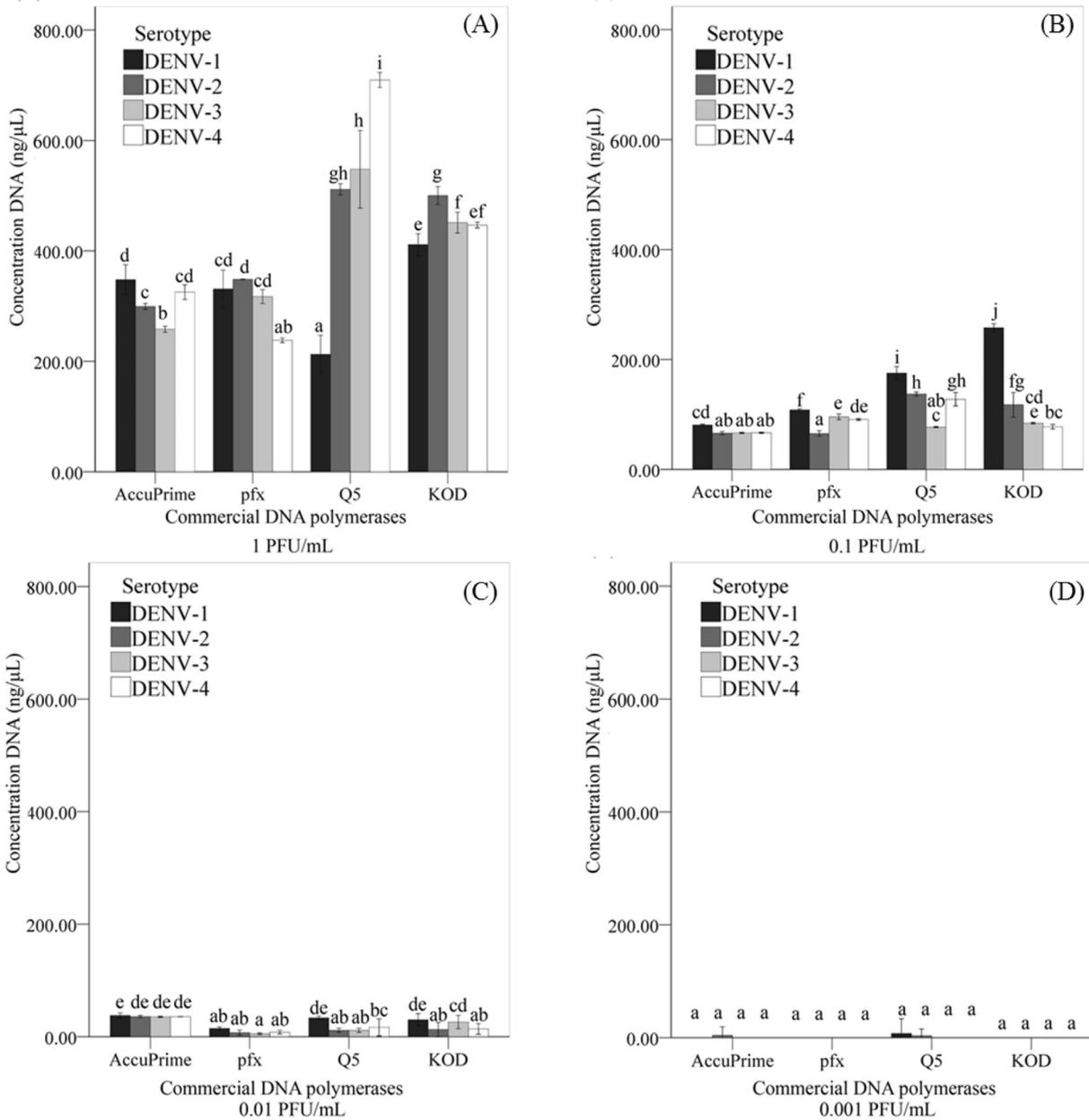
ANOVA and the Tukey's HSD test were performed using functions in the R programming language (R Development Core Team R., 2013).

#### Results

This project aimed to examine the efficiency of the four high-fidelity DNA polymerases (AccuPrime™ *Taq* DNA Polymerase High Fidelity, Platinum® Pfx DNA Polymerase, Q5® High-Fidelity DNA Polymerase and KOD FX Neo) in order to develop an efficient method for detecting and identifying DENV serotypes and genotypes from a sample with a very low viral load, especially in the field-caught mosquitoes. By examining these enzymes via the isolated DENV and DENV-infected mosquitoes, the results showed that both the sample types and the regions of DENV genome affected the efficiency of DNA polymerases.

By amplifying the C/prM junction from the DENV samples isolated from the viral cell culture, the agarose gel electrophoresis results (Fig. S1) showed that every DNA polymerase could amplify this region from every DENV serotype with at least 0.01 PFU/mL. AccuPrime and Q5 DNA polymerase could amplify this region from the DENV-2 sample with 0.001 PFU/mL; however, Q5 could also amplify this region from the DENV-1 sample. No non-specific PCR products were amplified. The productivity of these enzymes was examined by applying statistical tests to the PCR product concentrations. Regardless of the DENV serotype and type of DNA polymerase, the PCR product concentrations decreased with a reduction in the viral sample concentration, as shown in Fig. 1. The concentration of the PCR product of DENV-2 amplified by Q5 was not significantly different from that amplified by AccuPrime; in fact, the concentration of the PCR products amplified by Q5 or AccuPrime were not significantly different from zero (Fig. 1D). Hence, these results suggested that Q5 had the highest sensitivity compared to other enzymes included in this study.

As shown in Fig. 1A–C, the PCR products of different serotypes with the same concentration amplified by different enzymes



**Fig. 1.** Comparison of the polymerase chain reaction product concentrations of the C/prM junction of four dengue virus (DENV) serotypes amplified by four different commercially-available DNA polymerases (AccuPrime™, Platinum® Pfx, Q5® and KOD FX Neo), where DENV samples were isolated from the viral cell cultures and serially diluted generating four concentrations in plaque forming units (PFU)/mL: (A) 1.0; (B), 0.1; (C), 0.01; (D) 0.001 (different lowercase letters above columns indicate significant differences at  $p < 0.05$ ; error bars show  $\pm$ SE).

were significantly different for almost every pairwise comparison generating groups of samples that followed neither the type of DNA polymerase nor the DENV serotype. Regarding the viral samples with 1.0 PFU/mL, the concentrations of the PCR products amplified by AccuPrime and Pfx were relatively similar and significantly lower than those amplified by Q5 or KOD for every DENV serotype except for the PCR product of DENV-1 amplified by Q5, as shown in Fig. 1A. The relationship of PCR product concentrations amplified from the viral samples with 0.1 PFU/mL was different. As shown in Fig. 1B, the PCR product concentrations amplified using AccuPrime and Pfx were still relatively similar; however, they were only slightly lower than those amplified by Q5 or KOD. Interestingly, among the four DENV serotypes, the concentrations of the PCR products amplified from DENV-1 samples were the highest regardless of the type of DNA polymerase used to amplify them.

Unlike the viral samples with 1.0 PFU/mL and 0.1 PFU/mL, the concentrations of the PCR products amplified from the viral samples with 0.01 PFU/mL using AccuPrime were significantly different from those amplified by Pfx, Q5 or KOD as shown in Fig. 1C. Therefore, these results suggested that the type of DNA polymerase and the DENV serotype could affect the concentration of the final PCR products.

Due to its high sensitivity for detecting the DENV virus isolated from the viral cell culture, Q5 was chosen for screening the field-caught mosquitoes for DENV infection. The screening results showed that 16 out of 717 pooled samples were positive and all serotypes were observed in this study as shown in Table 2. Four DENV-positive samples—DENV-1 collected from Bangkok in 2015, DENV-2 collected from Nakhon Ratchasima in 2014, DENV-3 collected from Lopburi in 2015 and DENV-4 collected from

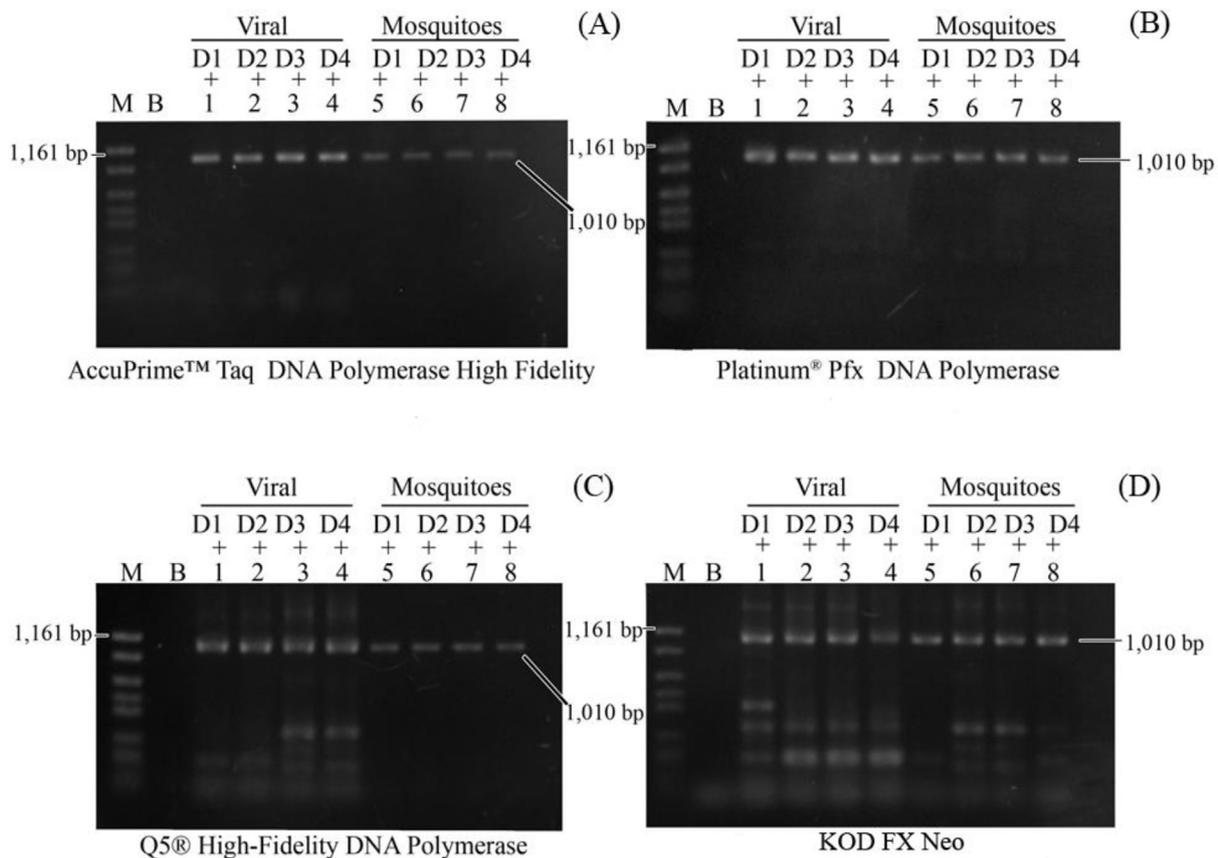
**Table 2**  
Numbers of pooled female *Aedes aegypti* mosquitoes collected from different geographical areas in Thailand during 2011–2015.

Sampling site	Number of pooled female <i>Aedes aegypti</i>	Number of positive mosquitoes separated by serotypes				Positive serotype samples (collection year)
		1	2	3	4	
<b>Central Thailand</b>						
Bangkok	143	2	–	5	–	DENV-1 (2015), DENV-3 (2011, 2015)
Pathum Thani	137	–	–	–	–	–
Nonthaburi	69	–	–	1	–	DENV-3 (2011)
Lopburi	22	–	–	2	–	DENV-3 (2015)
Suphanburi	15	–	–	–	–	–
<b>Eastern Thailand</b>						
Chanthaburi	49	–	–	–	1	DENV-4 (2013)
Chasengsao	7	–	–	–	–	–
Trat	7	–	–	–	–	–
<b>Northeast Thailand</b>						
Nakhon Ratchasima	111	–	1	–	–	DENV-2 (2014)
Southern Thailand						
Songkhla	157	–	–	4	–	DENV-3 (2012)
<b>Total</b>	<b>717</b>	<b>2</b>	<b>1</b>	<b>12</b>	<b>1</b>	<b>16</b>

Chanthaburi in 2013–were further studied to examine the efficiency of the four high-fidelity DNA polymerases in the amplification of the C/prM junction, the partial NS5 gene and the complete E gene.

The PCR products could be amplified from every selected DENV-positive pooled sample; thus the sensitivities of all four enzymes in amplifying C/prM junction, the partial NS5 gene and the complete E

gene from the field-caught mosquito samples were not different. It was further speculated that the concentration of viral load in the pooled field-caught mosquitoes collected in this study should be greater than 0.001 PFU/mL. This was supported by the results presented in Fig. S2 showing that the concentrations of the PCR products amplified from the mosquito samples were all greater than those amplified from the DENV isolated from the viral cell



**Fig. 2.** Agarose gel electrophoresis results of the partial NS5 gene amplification where this region was amplified from dengue virus (DENV) samples isolated from the viral cell culture (Lanes 1–4) and the field-caught mosquitoes (Lanes 5–8) using four DNA polymerases: (A) AccuPrime™ Taq DNA Polymerase; (B) Platinum® Pfx DNA Polymerase; (C) Q5® High-Fidelity DNA Polymerase; (D) KOD FX Neo (size of the polymerase chain reaction product was approximately 1700 bp; Lanes M and B represent the 100 bp DNA ladder and a negative control, respectively; Lanes D1–D4 represent DENV-1 to DENV-4, respectively).

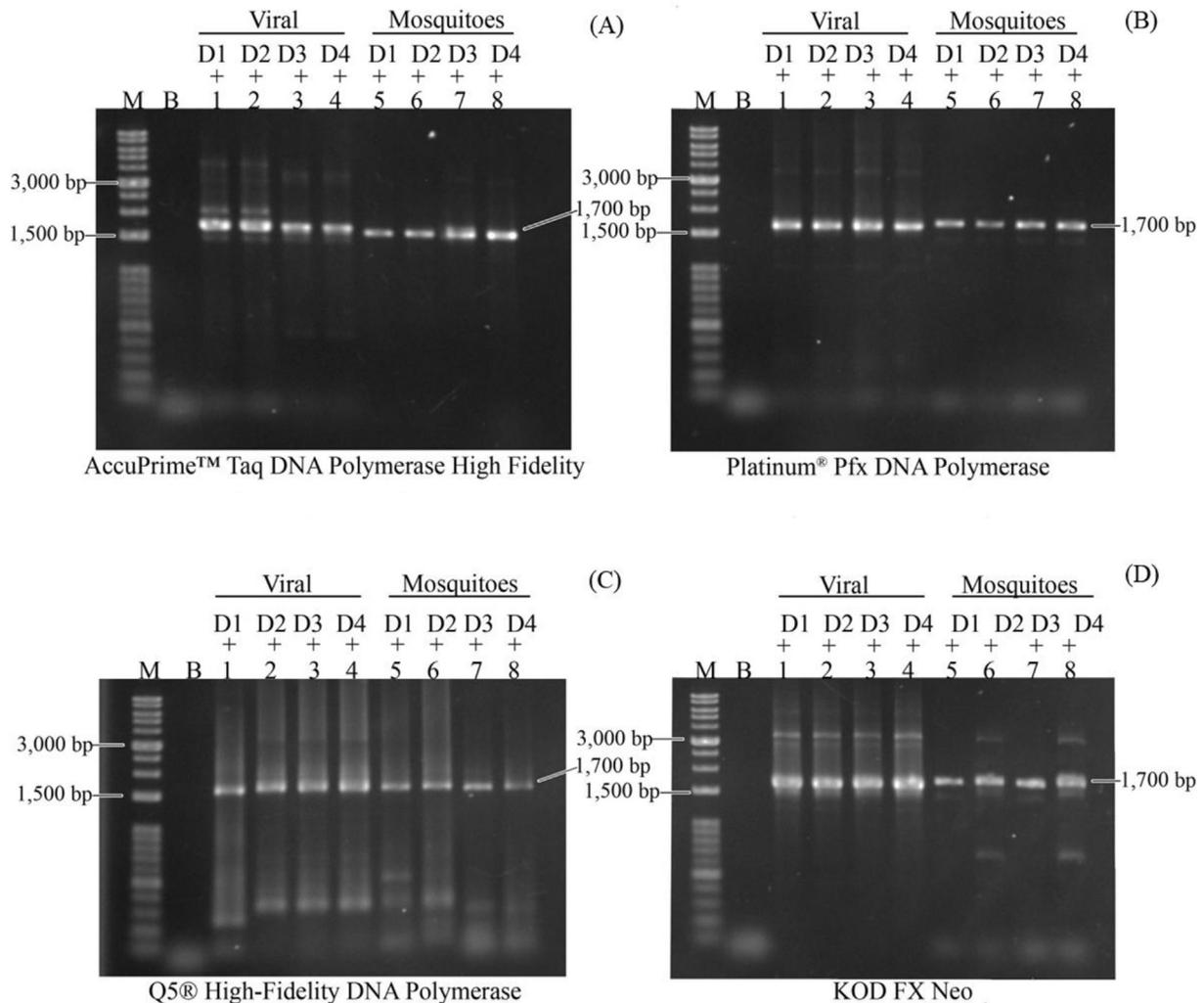
cultures with 0.1 PFU/mL. Regarding the specificity of the DNA polymerase, no non-specific PCR product was amplified from the C/prM junction of both isolated DENV samples and the field-caught mosquitoes as shown in Fig. S3. In the case of the partial NS5 gene, no non-specific PCR product was amplified using AccuPrime or Pfx but some non-specific PCR products were generated when this region was amplified using Q5 or KOD, as shown in Fig. 2. In particular, regarding the KOD enzyme, non-specific PCR products were amplified from both isolated DENV samples and the field-caught mosquitoes (Fig. 2D). In the case of the complete E gene, non-specific PCR products were generated from every DENV sample isolated from the viral cell cultures; however, no non-specific PCR products were amplified from the cDNA of the field-caught mosquitoes when this region was amplified using AccuPrime or Pfx, as shown in Fig. 3. Hence, these results suggested that the specificity of AccuPrime and Pfx was greater than for Q5 and KOD.

Regarding the productivity of the DNA polymerase, Pfx clearly provided the highest concentration of the PCR products of the C/prM junction regardless of the DENV serotype (Fig. 4A). In the case of the partial NS5 gene (Fig. 4B), KOD provided relatively high PCR product concentrations compared to the other enzymes. Following KOD, both AccuPrime and Pfx could also provide relative

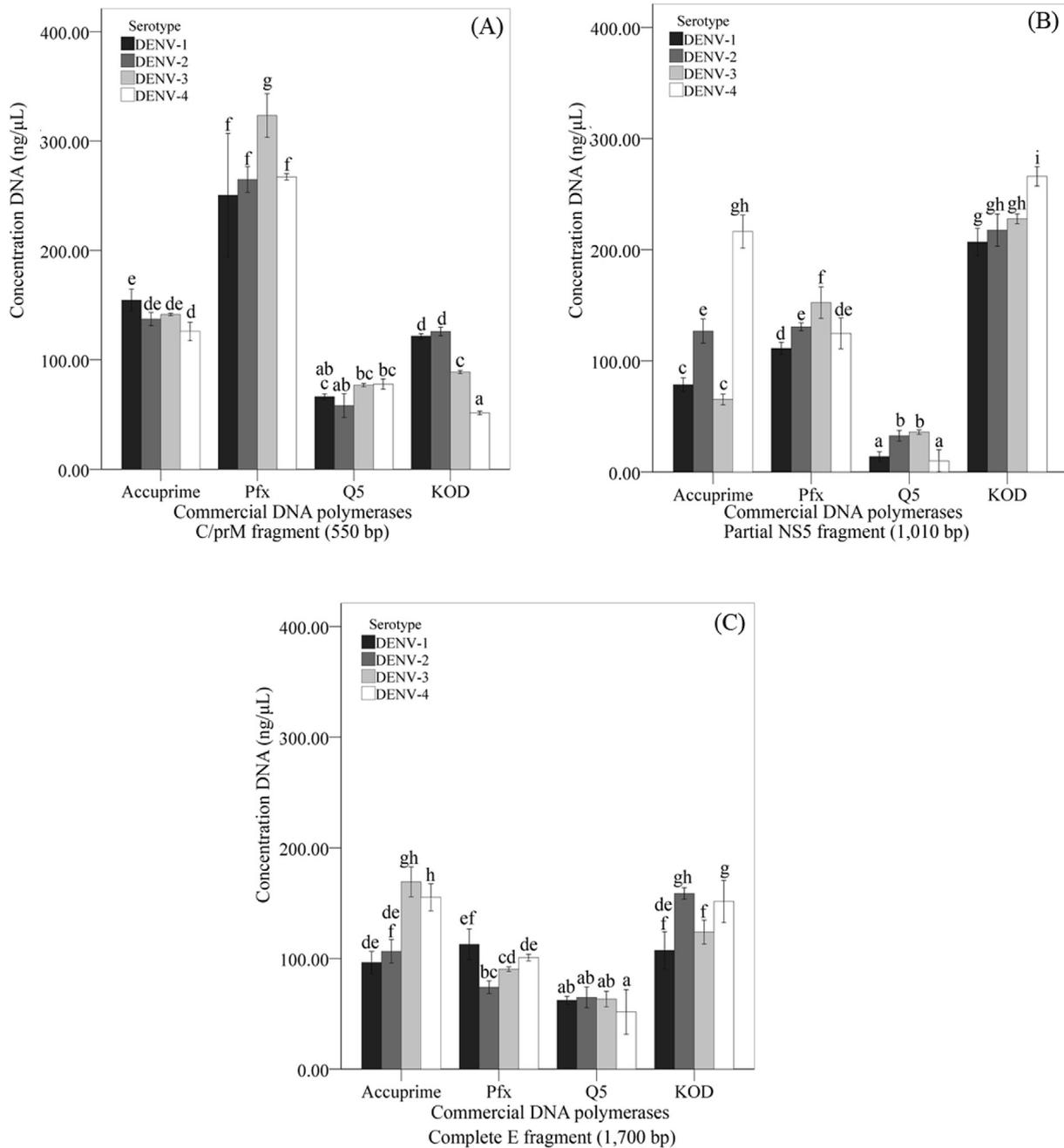
high concentrations of the PCR products. In the case of the complete E gene (Fig. 4C), AccuPrime and KOD provided relatively high concentrations of the PCR products. By taking into account the sensitivity, the specificity and the productivity of the DNA polymerase, Pfx and AccuPrime were the most efficient enzymes for amplifying C/prM and the complete E gene, respectively, and both enzymes had the greatest efficiency in amplifying the partial NS5 gene. These results suggested that different enzymes were suitable for amplifying different regions in the DENV genome.

**Discussion**

Early detection of DENV infection and identification of the serotype and genotype are crucial for diagnosis of dengue diseases (Peeling et al., 2010; Poloni et al., 2010). The method based on RT-PCR was highly sensitive for DENV detection and identification. The capability of this method depended on the efficiency of the DNA polymerase; therefore, this study evaluated the efficiency of DNA polymerase applied to amplify the C/prM junction and the NS5 and E genes. The results of this study suggested that the efficiency of different DNA polymerases depended on the type of samples and regions in the DENV genome.



**Fig. 3.** Agarose gel electrophoresis results of the complete E gene amplification. This region was amplified from dengue virus (DENV) samples isolated from the viral cell culture (Lanes 1–4) and the field-caught mosquitoes (Lanes 5–8) using four DNA polymerases: (A) AccuPrime™ Taq DNA Polymerase; (B) Platinum® Pfx DNA Polymerase; (C) Q5® High-Fidelity DNA Polymerase; (D) KOD FX Neo (size of the polymerase chain reaction product was approximately 1700 bp; Lanes M and B represent the 100 bp DNA ladder and a negative control, respectively; Lanes D1–D4 represent DENV-1 to DENV-4, respectively).



**Fig. 4.** Comparison of every dengue virus (DENV) serotype carried by field-caught mosquitoes of polymerase chain reaction product concentrations amplified from: (A) C/prM junction; (B) partial NS5 gene; (C) complete E gene; (four high fidelity DNA polymerases—AccuPrime™ Taq DNA Polymerase, Platinum® Pfx DNA Polymerase, Q5® High-Fidelity DNA Polymerase and KOD FX Neo—which were chosen to amplify these regions in the DENV genome (different lowercase letters above columns indicate significant differences at  $p < 0.05$ ; errors bars show  $\pm$  SE).

No non-specific PCR product of the C/prM junction was amplified from DENV isolated from the viral cell cultures; thus the specificity of every enzyme was similar. Regarding the sensitivity of the enzyme, Q5 and AccuPrime could amplify the C/prM junction from the DENV-2 sample with 0.001 PFU/mL, but only Q5 could amplify this region from the DENV-1 sample with this concentration; thus Q5 had the highest sensitivity. Interestingly, the sensitivity of the current method for detecting DENV by amplifying the C/prM junction was greater than other methods reported in previous studies because the current method could detect DENV in the samples carrying at least 0.01 PFU/mL, while the earlier methods could detect DENV from the samples with at least 0.1 PFU/mL

(Jittmittraphap et al., 2006; Maneekan et al., 2009). Regarding the productivity of the enzyme, the concentration of the PCR products amplified from the samples with at least 0.01 PFU/mL depended on both the type of DNA polymerase and the DENV serotype. Das et al. (2008) reported the influence of the DENV serotype on the detection limit. They showed that the detection threshold of DENV-2 was the lowest, while the highest detection threshold was observed in DENV-3. The concentration of the PCR products amplified from the samples with 0.001 PFU/mL using Q5 or AccuPrime were not significantly different from the no PCR product, which could have been a consequence of being unable to repeatedly amplify the C/prM junction of DENV-1 and DENV-2 using either of Q5 or

AccuPrime (Table S1). Regarding the consistency of the PCR product concentration, AccuPrime yielded relatively consistent concentrations among the four different DENV serotypes when the concentration of the viral samples was greater than 0.001 PFU/mL, as shown in Fig. 1A–C. Therefore, these results suggested that both AccuPrime and Q5 were suitable for screening DENV infection in the field-caught mosquitoes; however, due to the fact that the cost per sample of AccuPrime was much higher than that of Q5, Q5 was chosen for screening DENV infection in the field-caught mosquitoes.

The screening results showed that all four DENV serotypes had circulated in Thailand from 2011 to 2015 with the most frequent DENV serotype observed in this study being DENV-3. The current study observation regarding the circulation of all four serotypes corresponded well with the epidemiological report published by the Department of Disease Control, Ministry of Public Health, Thailand; however, DENV-3 was not the prevalent serotype, except for the year 2015 (Ketkaew et al., 2016). This difference could be explained by the small number of both the collecting sites and the mosquito samples explored in this study. Another explanation for this difference was that the infection rate observed in the field-caught-mosquitoes probably did not correspond well with the incidence rate of dengue diseases observed in humans as recently reported (Peña-García et al., 2016). Interestingly, among six samples collected from the Faculty of Fishery, Kasetsart University, Bangkok, Thailand in October 2015, four samples carried DENV-3 and two samples carried DENV-1. This observation indicated a co-circulation of these two DENV serotypes in a small area. This should be a concern because the co-circulation could generate a high incidence rate of dengue diseases in the area (Jarman et al., 2008; Fatima et al., 2011; Lee et al., 2012). The circulation of all four serotypes and the co-circulation of DENV-1 and DENV-3 observed in this study emphasized the need to develop an efficient method for DENV detection and serotype identification.

The sensitivities of every enzyme applied to examine the four pooled mosquito samples carrying different DENV serotypes were equal because they could amplify PCR products of every region from the mosquito samples. Due to the presence of non-specific PCR products amplified by Q5 and KOD, the specificity of AccuPrime and Pfx was greater than for the former two enzymes. The non-specific PCR products generated by KOD observed in this study corresponded well with a previous study that applied this DNA polymerase to detect another mosquito-borne disease by amplifying small-subunit rRNA genes using direct PCR (Miura et al., 2013). By taking into account the sensitivity, specificity and productivity of the enzyme, Pfx and AccuPrime would be the most efficient enzymes for amplifying the C/prM junction and the complete *E* gene from the mosquitoes, respectively. If the consistency of the PCR product concentrations across different DENV serotypes were taken into account, Pfx would be slightly better than AccuPrime in amplifying the partial *NS5* gene. The association between DENV genome regions and DNA polymerase efficiency could be explained by priming bias as different families of DNA polymerases preferred to bind different sequence motifs (Pan et al., 2007). Interestingly, in the case of the C/prM junction, Q5 was no longer considered as the most efficient enzyme when it was applied to amplify this region from the field-caught mosquitoes. The dependency of DNA polymerase efficiency on the type of samples observed in the current study was supported by a previous study that reported that different DNA polymerases were suitable for amplifying PCR products from samples containing PCR inhibitors and buried bone samples with degraded DNA (Nilsson et al., 2016). Hence, these results suggested that different DNA polymerases were suitable for amplifying different regions in the DENV genome extracted from different types of samples.

In conclusion, the results of this study suggested that Q5, Pfx and AccuPrime should be used to detect and identify DENV serotypes and genotypes; Q5 was suitable for detecting DENV isolated from viral cell cultures and for screening DENV in field-caught mosquitoes, while Pfx and AccuPrime were suitable for identifying the DENV serotype and genotype from field-caught mosquitoes. These results would be useful for developing an efficient method based on RT-PCR for DENV detection, and serotyping and genotyping from low viral load samples, especially with DENV-infected mosquito vectors.

### Conflict of interest

The authors declare that there are no conflicts of interest.

### Acknowledgements

This study was financially supported by the Graduate Program Scholarship from the Graduate School, Kasetsart University and the Kasetsart University Research and Development Institute (KURDI), Kasetsart University, Bangkok, Thailand (Grant No. 103.59). The authors thank Mr Wirat Wonghiranracha for his help regarding mosquito collection and identification. Help regarding mosquito collection was provided by officers of the Office of Vector Borne Disease Control Region 4 Songkhla province, Office of Vector Borne Disease Control Region 3.5 Chanthaburi province, Office of Vector Borne Disease Control Region 1 Bangkok, Department of Disease Control, Ministry of Health, Thailand and by staff at Na Yai Am Hospital, Chanthaburi province, Thailand.

### Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.anres.2018.05.012>.

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