การพัฒนาเทคนิคสำหรับการตรวจจับความแตกต่างของไวรัสชิคุนกุนยา สายพันธุ์ East/Central/ South Africa

Development of Differential Detection Chikungunya Virus in East/Central/ South Africa Lineage

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บทคัดย่อ

โรคชิคุนกุนยาเป็นโรคที่เกิดจากไวรัสชิคุนกุนยาโดยมียุงลายเป็นพาหะ ในแต่ละปีจะมีผู้ป่วยติดเชื้อไวรัสชิคุนกุนยา มากกว่าหนึ่งล้านคน สำหรับในประเทศไทยไวรัสชิคุนกุนยาถูกค้นพบครั้งแรกเมื่อปี 1958 หลังจากนั้นในปี 2008-2009 เกิดการระบาดอย่างรุนแรงของไวรัสชิคุนกุนยาสายพันธุ์ East/Central/South Africa ที่มีการกลายพันธุ์ในยีน E1 เป็นแบบ A226V ในการศึกษาครั้งนี้เพื่อวิเคราะห์ความหลากหลายทางพันธุกรรมของไวรัสชิคุนกุนยาที่พบประเทศไทย พร้อมทั้งพัฒนาวิธีการตรวจหาการกลายพันธุ์ของสายพันธุ์ ECSA ที่มีการระบาดอย่างรุนแรงด้วยวิธี multiplex PCR จากผลการวิเคราะห์ Phylogenetic tree พบว่าสายพันธุ์ของไวรัสชิคุนกุนยาที่พบในปี 2018-2019 ของประเทศไทยยังคงเป็นสายพันธุ์ ECSA ซึ่งเป็นแบบการกลายพันธุ์พร้อมกันสองตำแหน่งของ A226 กับ K211E ในยีน E1 นอกจากนี้ยังพบการกลายพันธุ์ในโปรตีน E2 เป็นแบบ V264A ดังนั้นทางคณะผู้วิจัยจึงได้ทำการออกแบบไพรเมอร์และหาสภาวะที่เหมาะสมในการทำ multiplex PCR เพื่อตรวจสอบ E1:A226, E1:K211E และ E2:V264A พบว่าสภาวะที่เติม 50, 100 หรือ 200 ng/µl DNA template, 1.5 mM $MgCl_2$, 10 mM Tris-HCL (pH 9.1), 0.2 mM dNTP, 0.25 µM of primer mix และ 2.5 Units DNA polymerase

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เป็นสภาวะที่เหมาะสมในการเพิ่มจำนวนชิ้นส่วนของ A226, K211E และ V264A ในขั้นตอนเดียว ซึ่งจากผลการทดลองนี้ชี้ให้เห็นว่าสามารถนำไปใช้ในการตรวจหาสายพันธุ์ ECSA ที่มีการระบาดอย่างรุนแรงได้อย่างง่ายและรวดเร็ว

คำสำคัญ: ไวรัสชิคุนกุนยา สายพันธุ์ East/Central/South Africa Multiplex PCR

Abstract

Chikungunya fever (CHIKF) is a mosquito-borne disease endemic to tropical regions. It is caused by the Chikungunya virus (CHIKV) and infects over one million people per year. CHIKV was first reported in Thailand in 1960 and a huge outbreak occurred in 2008-2009, which belonged to the East/Central/South Africa (ECSA) lineage (E1: A226V mutation). In this study, we analyzed the CHIKV E1 and E2 protein sequences in Thailand isolates and developed a method for detecting wild-type and mutant forms of the highly invasive ECSA lineage using one step multiplex PCR assay. A phylogenetic tree revealed that CHIKV found in Thailand during 2018–2019 represented the ECSA lineage and contained a double mutation (A226V and K211E) of the E1 protein. An additional E1 residue change, V264A, in the E2 protein of the ECSA lineage was also observed. Therefore, we designed specific primers and optimized multiplex PCR conditions to detect E1:K211E, E1:A266, and E2:V264A. The multiplex PCR results indicated that a PCR mixture containing 50, 100, or 200 ng/µl of DNA template, 1.5 mM $MgCl_2$, 10 mM Tris-HCL (pH 9.1), 0.2 mM dNTP, 0.25 µM of primer mix, and 2.5 units of DNA polymerase amplified A226, K211E, and V264A in one reaction. The results indicate that this assay can readily detect the ECSA strain in one reaction, which will facilitate the routine, high-throughput diagnosis of CHIKV.

Keywords: Chikungunya virus, East/Central/South Africa lineage, Multiplex PCR

Introduction

Chikungunya fever (CHIKF) is a mosquito-borne disease endemic to tropical regions. It infects over one million people each year (Yactayo, Staples, Millot, Cibrelus, & Ramon-Pardo, 2016). Chikungunya virus (CHIKV) belongs to the *Alphavirus* genus of the Togaviridae family and causes debilitating joint pain in humans characterized by fever, polyarthralgia, myalgia, rash, and headache

(Agarwal et al., 2019; Silva & Dermody, 2017). CHIKV is transmitted by the *Aedes* species of mosquitoes and is capable of causing an epidemic and an urban transmission cycle with high rates of infection (Silva & Dermody, 2017). The virus was first identified in 1952–1953 during an outbreak that occurred in the Makonde Plateau in the southern region of Tanzania (Silva & Dermody, 2017).

Thailand experienced an urban outbreak in Bangkok in 1960, and past outbreaks have occurred in the provinces of Prachinburi (1976), Surin (1988), Khon Kaen (1991), Loei and Phayao (1993), and Nakhon Si Thammarat and Nong Khai (1995) (Pulmanausahakul, Roytrakul, Auewarakul, & Smith, 2011). A huge outbreak was reported in 2008–2009 in the southern provinces of Thailand, and nearly 22,000 cases of CHIKF were diagnosed during the first 5 months of 2009 (Pulmanausahakul et al., 2011). From the initial outbreak in the Narathiwas province, CHIKV spread throughout most of the 43 provinces in Thailand and more than 46,000 cases were reported (Pulmanausahakul et al., 2011). CHIKF re-emerged in 2018 in several southern provinces of Thailand including Phuket, Songkhla, Pattani, and Narathiwas (Burean of vector bone disease, 2018). There were 3,560 CHIKV patients in 2008, which was a 357-fold increase compared with 2017 (Burean of vector bone disease, 2018). However, in 2021, a CHIKV outbreak primarily occurred in central, northeast, southern, and northern Thailand (Burean of vector bone disease, 2021).

The CHIKV genome consists of approximately 11,800 nucleotides containing two open reading frames, which encode four nonstructural proteins (nsP1-4) and five structural proteins (C, E3, E2, 6K, and E1) as shown in Figure 1. The genome has a short 5'-untranslated region and a longer 3'-untranslated region consisting of stem-loop structures and direct repeats that are believed to be associated with adaptation of the virus to mosquito hosts (Burt et al., 2017; Silva & Dermody, 2017). CHIKV consists of three lineages including the East/Central/South African (ECSA), West African, and Asian strains (Agarwal et al., 2019; Sharif et al., 2021).

The E1 gene possesses adequate information for phylogenetic classification, whereas the E2 gene is known to be immunodominant within its structural polyprotein (Patil et al., 2018). The ECSA genotype has a new variant with an alanine-to-valine mutation in the E1 protein (E1:A226V). It has spread in areas where *Aedes albopictus* is predominant and exhibits increased stability, fitness, and infectivity (Agarwal, Sharma, Sukumaran, Parida, & Dash, 2016; Chansaenroj et al., 2020). When the lysine-to-glutamate mutation in the E1 protein (E1:K211E) and valine-to-alanine mutation in the E2

protein (E2:V264A) are present together with E1:A226 (wild-type), increased infectivity, dissemination, and transmission of the virus by *Aedes* mosquitoes occur, especially in *Aedes aegypti*. However, they do not affect viral fitness when they are present together with E1:A226V (mutant) (Agarwal et al., 2016; Chansaenroj et al., 2020). The ECSA lineage containing E1:A226V, known as the Indian Ocean Lineage (IOL), was reported during 2007-2010 in India. However, CHIKV strains identified from 2011 onward belong to the ECSA genotype rather than IOL (Agarwal et al., 2019; Sharif et al., 2021).

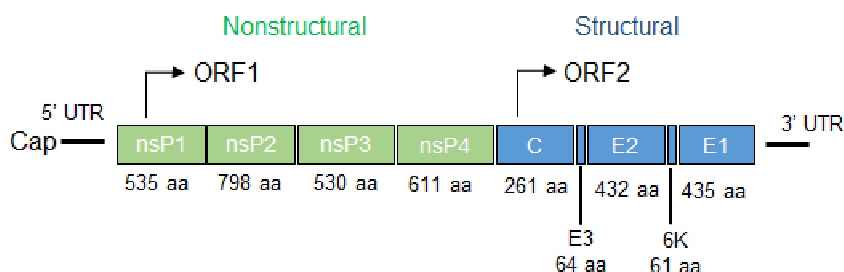

Figure 1 Organization of the chikungunya virus genome. The figure shows the position of the nonstructural and structural proteins in the genome and non-translatable regions in 5' and 3'. nsP, nonstructural protein; C, capsid; E, envelope glycoprotein; 6K, 6K viroporin channel; ORF, open reading frame; UTR, untranslated region; aa, amino acid.

The previous CHIKF outbreaks in Thailand during 2008-2009 were caused by CHIKV, the ECSA genotype containing *E1*:A226V (Suangto, 2009). In 2018, CHIKV isolated from an outbreak in southwest Bangkok showed that mutations in the predicted E1:K211E and E2:V264A proteins were identified in CHIKV-infected patients and in the *Aedes aegypti* mosquito (Chansaenroj et al., 2020). Recently, in 2019, the CHIKV genome was detected in *Ae. aegypti* collected from endemic areas of Thailand and contained the E1:A226V mutation, which was similar to the sequences isolated from the serum of patients in the previous outbreaks in Thailand, Malaysia, Korea, Singapore, India, and Sri Lanka during 2008-2010. The E1:K211E mutation was also observed (Intayot et al., 2019). Diagnosis of CHIKV infection is done by various clinical, epidemiological, and laboratory methods. Molecular assays, such as RT-PCR, RT-LAMP, and qRT-PCR, are the most reliable methods for diagnosing CHIKV due to the high sensitivity and specificity (Sharif et al., 2021). However, no molecular techniques have been reported that can differentiate wild-type and mutant CHIKV strains. To manage

epidemics in the future, the development of effective routine diagnostic assays is required to reduce the risk of future outbreaks and associated health burdens. Therefore, in this study, we developed a new multiplex PCR assay for the specific detection of E1:A266 wild type and E1:K211E, E2:V264A mutations which represent an easy and high-throughput method for routine diagnosis of CHIKV.

Objectives

1. To analyze the CHIKV E1 and E2 protein sequence in Thailand isolates

2. To develop a multiplex PCR assay for the detection of E1:A266 wild type and E1:K211E, E2:V264A mutations

Research Methodology

1. Phylogenetic analysis

Partial E1 protein sequences were used as BLAST queries to search the National Center for Biotechnology Information (NCBI) database and were aligned using the ClustalW program of BioEdit. Protein sequences were primarily selected from Thailand and Southeast Asia to understand the CHIKV epidemic in Thailand. Phylogenetic trees were constructed using Molecular Evolutionary Genetics Analysis (MEGA, version 10) software (Kumar et al., 2018). The maximum-likelihood method and the JTT (Jones-Taylor-Thornton) model were applied according to a phylogenetic model analysis (Kumar et al., 2018). Bootstrap resampling analysis consisting of 1000 replicates was performed.

2. CHIKV E1 and E2 gene construction and primer design

The E1 and E2 gene sequences for the ECSA genotype were obtained from the NCBI database (accession number: MK468801.1). The E1 and E2 genes (909 nucleotides) were custom-synthesized and cloned into the pET28b vector (Genscript, USA). The pET28-E1-E2 plasmid was transformed into *Escherichia coli* DH5α competent cells using a heat shock method. The pET28-E1-E2 cells were grown in Luria-Bertani broth supplemented with 50 µg/ml kanamycin and incubated at 37 °C and 170 rpm overnight. The plasmid was extracted from *E. coli* cells using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, USA) and used as a DNA template for PCR

amplification. For designing primer, we use the sequence of CHIKV ECSA lineage (MK468801.1) as the template. The primer sequences for E1:K211E, E1:A226, and E2:V264A are listed in Table 1.

Table 1 Wild-type and mutant primer sequences specific for the E1 and E2 genes of the ECSA lineage

Primer	Sequence (5'→3')	Tm	Amplicon (bp)	Target gene	Type
K211E-F	GTC GCA CAC CTG AGA GCG AA	55.9	311	E1	MT
K211E-R	ATG GGT GCA GGC TGG TAC CT	55.9			
A226-F	CTG CAG AGA CCG GCT GCG	57.2	417	E1	WT
A226-R	GGC CGT CGA GAA AGA GAT TTG C	56.7			
V264A-F	CCG TTT CCG CTG GCA AAT GCA	56.3	204	E2	MT
V264A-R	CCC TTC GGT CGG CAC GG	56.7			

3. PCR amplification

The PCR conditions to amplify the E1:K211E, E1:A226, and E2:V264A fragments were optimized. Each 20 µl PCR mixture contained 200 ng/µl DNA template, 1.5 mM $MgCl_2$, 10 mM Tris-HCL (pH 9.1), 0.2 mM dNTP, 2 µM of each primer (Table 1), and two units of Taq DNA polymerase (Vivantis). The PCR amplification was performed using a T100 Thermal Cycler (Bio-Rad). The pre-denaturation step was 2 min at 94 °C. Then, 35 subsequent cycles were done for 30 s at 94 °C, 30 s at 52 °C or 56 °C, and 30 s at 72 °C, followed by a final extension step for 7 min at 72 °C. The amplicons for E1:K211E, E1:A226, and E2:V264A were analyzed by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

4. Optimization of multiplex PCR

For multiplex PCR amplification, each reaction mixture contained the components listed in Table 2. The primer mix for each reaction consisted of K211E-F, K211E-R, A226-F, A226-R, V264A-F, and V264A-R. The annealing temperatures (Tm) varied and were tested at 62.0, 61.2, 60.0, 58.1, 55.8, 53.9, 52.7, and 52.0 °C. The PCR products were electrophoresed for 30 min at 80 V in 1× TBE buffer, and the gels were stained with ethidium bromide and visualized under UV light.

Table 2 Optimized PCR reaction components

Components	PCR reaction (final concentration)					
	I	II	III	IV	V	VI
10X PCR buffer	1X	1X	1X	1X	1X	1X
Template DNA	50 ng	50 ng	100 ng	100 ng	200 ng	200 ng
Primer mix	0.1 µM	0.25 µM	0.1 µM	0.25 µM	0.1 µM	0.25 µM
dNTP	0.2 mM	0.2 mM	0.2 mM	0.2 mM	0.2 mM	0.2 mM
$MgCl_2$	1.5 mM	1.5 mM	1.5 mM	1.5 mM	1.5 mM	1.5 mM
Taq DNA polymerase	2.5 Units	2.5 Units	2.5 Units	2.5 Units	2.5 Units	2.5 Units

Results

1. Phylogenetic analysis

Phylogenetic analysis using the maximum-likelihood method classified the CHIKV into three genotypes: ECSA, Asian, and West African as shown in Figure 2. Here we analyzed 20 partial E1 proteins that included CHIKV Thai strains during 1995 – 2019 and, partial CHIKV E1 proteins found in Southeast Asia for understating the evolution history and epidemic of CHIKV in Thailand. Phylogenetic analysis revealed that CHIKV was present in Thailand during 1958–1988 and belonged to the Asian lineage containing E1:K211E, E1:A226, and E2:V264A (Table 3). However, CHIKV in Thailand isolated in 2009–2013 belongs to the ECSA genotype that contained E1:K211, E1:A226V, and E2:V264A. Interestingly, an outbreak of CHIKV infection occurred in southwest Bangkok during 2018–2019 that belonged to the ECSA genotype containing E1:K211E, E1:A226, and E2:V264A and showed similarities to the strains found in Thailand during 1958–1988 (Figure 2, Table 3) (Chansaenroj et al., 2020). Double mutant: E1K211E, E2A226V in the background A226 (WT) revealed markedly higher fitness for *Ae. aegypti*, as evidenced by a significant increase in virus infectivity (13-fold), dissemination (15-fold), and transmission (62-fold) compared with the parental virus (Agarwal et al., 2016). These mutations occur in Thailand during 2018–2019. Therefore, the design of primers specific to these mutations can manage and prevent CHIKV epidemics in the future.

The superimposition of non-mutated and mutated E1 and E2 protein was reported in a previous study, and it showed that the mutation in the E1 protein did produce a structural difference,

whereas the moderate structural difference in domain A of E2 protein (V264A) was observed (Agarwal et al., 2016). The residues in this study (A226V, K211E, and V264A) are highlighted in the 3D structure shown in Figure 3.

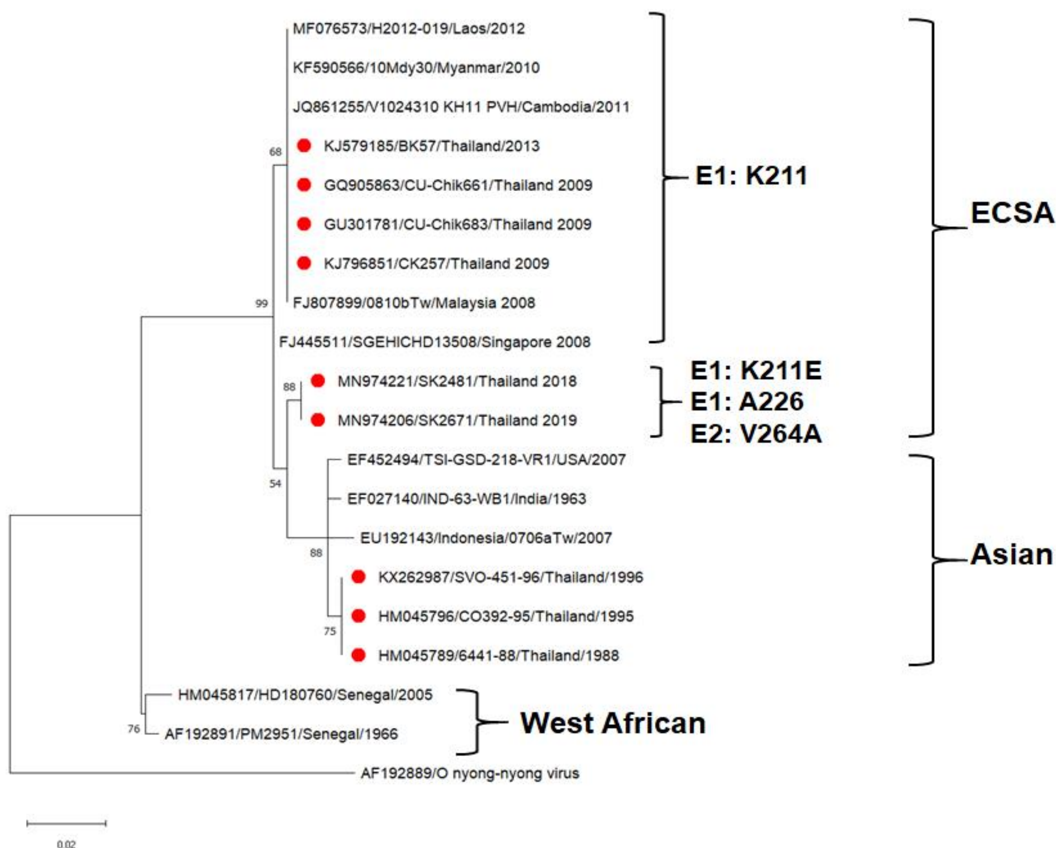

Figure 2 Phylogenetic tree of chikungunya virus strains performed using the partial E1 protein. The maximum-likelihood method with a bootstrap value of 1,000 was applied using the JTT model. The chikungunya virus Thai is marked with a symbol. The O'nyong nyong virus (AF192889) sequences were used as an outgroup. Viruses were identified using the GenBank accession number/strain/country/year of isolation. The scale bar at the bottom indicates the genetic distance in nucleotide substitutions per site. E, envelope glycoprotein; ECSA, East/Central/South Africa lineage; JTT, Jones-Taylor-Thornton model.

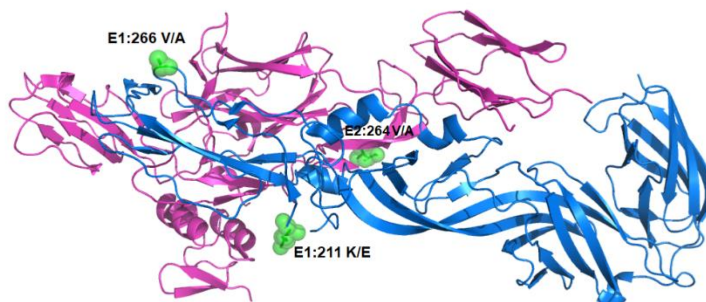

Figure 3 Structure representation of chikungunya virus E1-E2 heterodimer (PDB:3N41). E1 and E2 proteins are cartoon diagrams in blue and magenta color, respectively. The mutated residue used in this study is represented in sphere representation. E, envelope glycoprotein; PDB, protein data bank.

Table 3 Comparison of amino acid substitutions in the E1 and E2 protein sequences from humans infected with CHIKV in Southeast Asia

GenBank accession	Strain	Collection Year	Country	Amino acid Position						
				E1:211	E1:226	E1:269	E1:284	E1:340	E1:349	E2:264
LC259082	BaH306-NIID	1958	Thailand	E	A	M	D	R	N	A
HM045810	TH35	1958	Thailand	E	A	M	D	R	N	A
HM045789	6441-88	1988	Thailand	E	A	M	D	R	S	A
HM045787	SV0444-95	1995	Thailand	E	A	M	D	R	S	A
FJ807896	0611aTw	2006	Singapore	K	A	V	E	R	N	A
EU703759	MY002IMR/06/BP	2006	Malaysia	E	A	M	D	R	N	A
EU703760	MY003IMR/06/BP	2006	Malaysia	E	A	I	D	R	N	A
EU703761	MY019IMR/06/BP	2006	Malaysia	E	A	M	D	W	N	A
FN295483	MY/06/37348	2006	Malaysia	E	A	M	D	R	N	A
FN295484	MY/06/37350	2006	Malaysia	E	A	M	D	R	N	A
FJ807897	0706aTw	2007	Indonesia	E	A	M	D	R	N	A
FJ807899	0810bTw	2008	Malaysia	K	V	V	E	R	N	A
FN295487	MY/08/068	2008	Malaysia	K	V	V	E	R	N	A
FJ445432	SGEHICHS422308	2008	Singapore	K	V	V	E	R	N	A
FJ445445	SGEHICHS425208	2008	Singapore	K	V	V	E	R	N	A
FJ445484	SGEHICHT077808	2008	Singapore	K	V	V	E	R	N	A
GQ905863	CU-Chik661	2009	Thailand	K	V	V	E	R	N	A
KT324225	MY/09/4493	2009	Malaysia	K	V	V	E	R	N	A

Table 3 (Continue)

GenBank accession	Strain	Collection Year	Country	Amino acid Position						
				E1:211	E1:226	E1:269	E1:284	E1:340	E1:349	E2:264
KF151175	Myanmar/D136/2009	2009	Myanmar	K	V	V	E	R	N	V
KF590566	10Mdy30	2010	Myanmar	K	V	V	E	R	N	V
JQ861256	V1024311_KH11_PVH	2011	Cambodia	K	V	V	E	R	N	A
KJ579185	BK57	2013	Thailand	K	V	V	E	R	N	A
KJ579186	BK63	2013	Thailand	K	V	V	E	R	N	A
KJ579187	BK68	2013	Thailand	K	V	V	E	R	N	A
MH329300	Car-128	2014	Colombia	E	A	M	D	R	N	A
MN974221	SK2481	2018	Thailand	E	A	V	E	R	N	A
MN974206	SK2671	2019	Thailand	E	A	V	E	R	N	A

2. Agarose gel electrophoresis

The specificity of the primers (Table 1) used to detect E1:K211E, E1:A226, and E2:V264A in the ECSA strain. After PCR amplification, the result of agarose gel electrophoresis (Figure 4) suggested that the K211E, A266, and V264A primers, at annealing temperatures of 52 and 56 °C, were amplified products of approximately 311, 417, and 204 bp, respectively. Therefore, the ability of these primers to detect E1:K211E, E1:A226, and E2:V264A was demonstrated.

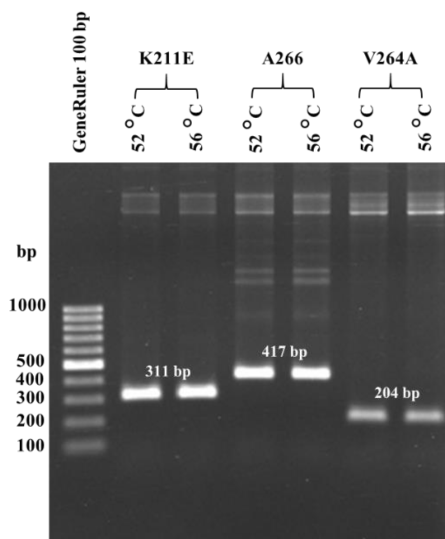

Figure 4 1.5% agarose gel electrophoresis of the K211E, A266, and V264A PCR reaction products

After confirming the specificity of the primers for E1:K211E, E1:A226, and E2:V264A, a multiplex PCR assay was optimized for the detection of the three fragments in one tube. The six reaction components (Table 2) amplified PCR products at an annealing temperature gradient at 62.0, 61.2, 60.0, 58.1, 55.8, 53.9, 52.7, and 52.0 °C. The results of multiplex PCR (Figure 5A) indicated that PCR reactions I, III, and V (using 0.1 μM primer mix) detected E1:A226 and E1:K211E, whereas they could not detect E2:V264A. PCR reactions II, IV, and VI (using 0.25 μM primer mix) detected three products representing E1:A226, E1:K211E, and E2:V264A (Figure 5B-D). Moreover, reaction II is suitable condition that can detect three PCR products using 50 ng of template.

Discussion

The Chikungunya virus (CHIKV) causes Chikungunya fever, an arthropod-borne viral disease transmitted by the *Aedes aegypti* and *A. albopictus* mosquitoes. The Chikungunya fever is a major public health problem in several areas of Africa, South America, and Southern and Southeast Asia (Pulmanausahakul, et al., 2009). In Thailand, CHIKV outbreaks were first documented in early 1958 and represented the Asian lineage. A huge outbreak was reported in 2008–2009, which had the highest case rate of approximately 49,000 people (Ganesan, Duan, & Reid, 2017), especially in Narathiwas. This outbreak involved CHIKV strains belonging to the ECSA lineage, and most of the infected cases contained the E1:A226V mutation (Rianthavorn, Prianantathavorn, Wuttirattanakowit, Theamboonlers, & Poovorawan, 2010). Recently (2013–2019), CHIKV that spread throughout Thailand was found to contain A226 and K211E in Bangkok patients (Chansaenroj et al., 2020) and double mutations (A226V and K211E) in the E1 protein in *Ae. aegypti* (Intayot et al., 2019). An additional E1 protein residue change (E2:V264A) in the ECSA lineage was also observed in Bangkok patients (Chansaenroj et al., 2020). A previous study revealed that the E1:A226V mutation enhances transmission and dissemination, whereas the double mutant E1:K211E, E2:V264A and the background E1:A226 (WT) increase infectivity, transmission, and dissemination by *Ae. aegypti* (Agarwal et al., 2016). Therefore, the identification of CHIKV strains with distinctive signature residues may assist in understanding the global viral movement and transmission pattern of this virus, which are important for reducing its occurrence.

Figure 5 1.5% agarose gel electrophoresis of the multiplex PCR reactions III (A), II (B), IV (C), and VI (D). Neg A is negative control for reactions II, IV, VI, whereas Neg B is negative control for I, III, V (E).

The detection of CHIKV is done by several methods, such as virus propagation, reverse transcriptase polymerase chain reaction (RT-PCR), multiplex real-time PCR, commercial rapid test, ELISA, HI test, and antibody screening (IgM, IgG). Furthermore, a previous study showed that the detection of CHIKV can be performed together with dengue virus (DENV) using multiplex real-time PCR with high sensitivity and specificity (Pongsiri, Praianantathavorn, Theamboonlers, Payungporn, & Poovorawan, 2012). Antibody screening generally results in false positives because IgM against CHIKV may cross-react with other alphaviruses, such as the O'nyong nyong virus. Virus culture has shown reliable results but is laborious and time-consuming to perform (Pongsiri et al., 2012). Nonetheless, there is currently no method for the rapid detection of the strains and mutations of CHIKV. Multiplex polymerase chain reaction (PCR) can amplify two or more target DNA fragments using more than one pair of primers in the same reaction. This technique has been successfully adapted for mutation and polymorphism analysis. Multiplex PCR has been applied to the identification of viruses, bacteria, and parasites in infectious diseases (Markoulatos, Siafakas, & Moncany, 2002). In this study, we optimized the conditions for a multiplex PCR assay to rapidly detect E1:K211E, E1:A266, and E2:V264A of ECSA CHIKV in a single tube. We successfully obtained suitable conditions for a multiplex PCR assay for the routine diagnosis of CHIKV.

Conclusions

A phylogenetic tree showed that CHIKV present in Thailand in 2018–2019 was ECSA. E1:K211E, E1:A226, and E2:V264A exhibit markedly higher fitness for *Ae. aegypti*, as evidenced by a significant increase in virus infectivity, dissemination, and transmission. Therefore, we designed specific primers to detect these mutations. Our primers and multiplex PCR conditions enabled the detection of E1:K211E, E1:A226, and E2:V264A. This method represents a useful tool for the early detection of CHIKV strains in one step for high-throughput, routine diagnosis during an epidemic.

Suggestion

These experiments represent preliminary results. Further RT-PCR studies are needed to establish the sensitivity and specificity of the assay for the Chikungunya genome. Additional experiments on human and mosquito samples will also be required.

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