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Entire genome characterization of Chikungunya virus from the 2008-2009 outbreaks in Thailand

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Abstract. The resurgence of Chikungunya virus (CHIKV) in the southern, northeastern and northern parts of Thailand, inflicting approximately 46,000 reported cases since October 2008 until December 2009, has raised public health concerns. In the present study, we characterized nearly complete genome sequences of four CHIKV isolates obtained from 2008 to 2009 outbreaks in Thailand. Phylogenetic analysis was performed to determine the relationships of the study viruses with previously reported isolates. Results showed that 2008-2009 Thailand isolates belonged to the East, Central and South African genotype and were most closely related to isolates detected in Malaysia and Singapore in 2008. This was in contrast to isolates from all previous outbreaks in Thailand which were caused by an Asian genotype. We describe several novel mutations in Thailand isolates that warrants further investigation on characterization of CHIKV from different parts of the country to better understand the molecular epidemiology of Chikungunya fever outbreaks in Thailand.

INTRODUCTION

Chikungunya Virus (CHIKV) is an enveloped, single-stranded RNA virus of positive polarity with a genome of ≈11.8 kb (Khan *et al.*, 2002) and belongs to the family *Togaviridae* and genus *Alphavirus* (Strauss & Strauss, 1994). The 5' two-thirds of the genomic RNA encode the non-structural proteins while the 3' one-third serves as mRNA for the synthesis of viral structural proteins (Faragher *et al.*, 1988).

CHIKV is responsible for chikungunya fever (CHIKF), of which main clinical features are fever, chills, headache, myalagia, maculopapular rash and arthralgia (Mohan, 2006). Although many of these features are non specific, arthralgia seems to be more specific to CHIKF than any other clinical findings (Rianthavorn *et al.*, 2010). Phylogenetic analysis of the CHIKV genome based on partial *E1* gene sequences has identified three genotypes of CHIKV; West African, Asian and East, Central and South African (ECSA) genotypes (Powers *et al.*, 2000). CHIKV was first isolated from a febrile patient during an epidemic of dengue-like illness in Tanzania (previously Tanganyika) in 1952–1953 (Robinson, 1955).

The virus is understood to have come from Africa and was subsequently introduced to many regions of Asia (Carey, 1971). The first isolation of CHIKV in Asia was reported from Thailand in 1958 (Hammon & Sather, 1964). Since 2004, several outbreaks have occurred with millions of reported cases, involving new geographical areas (Powers & Logue, 2007) such as Kenya in 2004, Comoros in 2005

(Sergon *et al.*, 2007), and several Indian Ocean islands in 2005 (Schuffenecker *et al.*, 2006). Furthermore, India reported an outbreak of unprecedented magnitude with more than 1.38 million cases in 2006-2007 (Lahariya & Pradhan, 2006; WHO, 2008). Furthermore, CHIKV has caused large epidemics in Southeast Asia with cases reported in Indonesia in 2001-2008(WHO, 2008), Singapore in 2008 (Ng *et al.*, 2009), Malaysia in 2006 (AbuBakar *et al.*, 2007) and Thailand in 2008 (Ungchusak, 2008). Virus strains circulating in Asia were of the Asian genotype until a shift to the ECSA genotype occurred in India in late 2005.

In Thailand, outbreaks of CHIKV have occurred in Prachinburi (1976), Surinn (1988), Khon Khen (1991), Loei & Prayao (1993), and Nongkhai & Nakorn Sri Thammaraj (1995). From late September to October 2008, CHIKF outbreaks occurred in many provinces of Southern Thailand. The initial outbreaks spread over five villages, affecting 82 individuals, the majority of whom used to travel regularly to Malaysia (Bureau of Epidermiology, Department of Disease Control, Thailand, 2008). The area mostly affected by CHIKV was Narathiwat, the Southernmost province of the country. There are plenty of *Aedes albopictus*, the vector of CHIKV, in plantation areas abundant in Southern Thailand, from where CHIKV was isolated from *Ae. albopictus* during the initial outbreaks (Ungchusak, 2008). We have already reported the clinical and viral molecular characteristics of initial CHIKF outbreaks in Thailand (Theamboonlers *et al.*, 2009). The same study indicated that CHIKV strains involved in the initial outbreaks in Thailand belonged to the ECSA genotype reported from the rest of Southeast Asia after 2005. These isolates harbored the E1-A226V mutation (Theamboonlers *et al.*, 2009; Rianthavorn *et al.*, 2010) that is known to increase the virus transmissibility via *Ae. albopictus* (Tsetsarkin *et al.*, 2007). By December 2009, CHIKV spread to the Central (17 provinces), Northeastern (15 provinces) and Northern (11 provinces) parts of the country affecting more than 46,000 individuals. The estimated rate of infection was 75.40 people per 100,000 people (Bureau of Epidemiology, Department of Disease Control, Thailand, 2008).

In this study, we established nearly the whole genome sequence of CHIKV detected in four serum samples obtained in 2008 and 2009. In addition, the phylogenetic origin and the diversity of CHIKV strains responsible for the re-emergence of CHIKF in Thailand were examined.

MATERIALS AND METHODS

Sample Collection

An outbreak of CHIKF was reported in the Southern Thailand in 2008. Samples were randomly picked up from sera that were tested positive for CHIKV by RT-PCR. We randomly selected one positive serum sample (CU-Chik10) reported in 2008. In 2009, with the outbreaks still ongoing, we received more sera and selected 3 serum samples for genetic characterization; two (CU-Chik661 and CU-Chik683) from Narathiwat, and one serum sample, CU-Chik009, from a patient who had returned from Nakhonsrithammaraj. Both areas were provinces in the Southern Thailand. Sample details are shown in Table 1. The details and molecular characterization of *E1* gene have been reported elsewhere (Theamboonlers *et al.*, 2009, Rianthavorn *et al.*, 2010).

Cell preparation

African green monkey, *Cercopithicus aethiops* kidney cells, Vero cells, and Rhesus monkey kidney, *Macaca mulatta*, LLC-MK_2 cells were used to propagate the virus from serum samples. These cells were maintained in growth medium, DMEM (Sigma, USA), supplemented with 10% heat inactivated (56°C for 30 min.) fetal bovine serum (Sigma, USA), 1.1 g/l sodium bicarbonate, 2mM L-glutamine, and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin). Cells were incubated at 37°C with 5% CO_2 in a humidified CO_2 incubator.

Virus propagation

CHIKV infected serum samples were diluted 1:5 in RNAse free PBS solution (final pH 7.2–7.4) before grinding in RNAse free PBS solution (final pH 7.2–7.4) and filtered through a 0.22 µm filter. Only the CU-Chik683 sample was propagated in Vero cell culture and was passaged once before sequencing. The rest of analyzed isolates (n=3) were sequenced directly from sera. Briefly, confluent monolayer Vero cells, approximately $5x10^5$ cells per T_{25} flask, were inoculated with prepared samples for 90 min at 37ºC on a rocker platform. The monolayer infected Vero cells were maintained in growth medium, DMEM (Sigma, USA), supplemented with 10% heat inactivated (56ºC for 30 min) fetal bovine serum (Sigma, USA), 1.1 g/l sodium bicarbonate, 2mM L-glutamine, and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin), and incubated at 37ºC with 5% CO_2 in a humidified CO_2 incubator. Once 70-80% cytopathic effect (CPE) was observed – approximately 5-7 days post inoculation – the culture supernatants were collected into bio-freeze vials and kept at -80ºC for viral RNA detection by RT-PCR and viral titration assay by plaque titration on LLC-MK2 cells.

Plaque titration assay

LLC-MK2 cells were used for the plaque assay, modifying the protocol originally described by Malewicz & Jenkin (1979). Briefly, LLC-MK2 cells were maintained in DMEM (Sigma, USA), supplemented with 10% heat inactivated (56ºC for 30 min) fetal bovine serum (Sigma, USA). Ten-fold dilutions of the infected Vero cell supernatant were diluted in 5% RPMI-1640 serum free medium (Sigma, USA). Subsequently, 70-80% confluent LLC-MK2 cells in a 6 well culture plate were washed once with Hanks' balanced salt solution and then inoculated with 250 µL of ten-fold diluted samples at 37ºC for 90 min. After that, the first overlay agar medium was incubated at 37ºC with 5% CO_2 in a humidified CO_2 incubator for 3-5 days until plaque formation. The second overlay containing 4% neutral red was added and incubated as above for 24 hours for plaque identification.

Whole genome characterization

RNA extraction and RT-PCR

CHIKV was isolated directly from the patient's sera (n=3) or from cell cultures (n=1) originating from the first passage Vero cells (Table 1). Viral RNA was extracted using the Viral Nucleic Acid Extraction Kit (RBC Bioscience, Taiwan) according to the manufacturer's protocol and amplified by reverse transcription polymerase chain reaction (RT-PCR), using the Superscript III platinum One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA). The reaction mixture consisted of 2 µl of extracted RNA, 5 µl of 2x reaction mix, 0.25 µl of superscript reverse transcriptase III platinum *Taq* polymerase, 0.5 µM of each primer, and 6 µl of nuclease-free water. The RT step and PCR amplification were performed in an Eppendorf Mastercycler personal (Eppendorf, Hamburg, Germany) under the following conditions: reverse transcription at 50 C for 30 min; subsequently, initial denaturation at 95ºC for 3 min; followed by 40 cycles of denaturation at 95ºC for 1 min, primer annealing at 55ºC for 1 min, and extension at 72ºC for 2 min; and concluded by final extension at 72ºC for 7 min. All primers, except for the reverse primer of fragment 21, are previously published (Schuffenecker *et al.*, 2006). The sequence of the newly designed reverse primer of fragment 21 was 5'CTCCTACGTCCCTGTGGG3'. The amplified PCR products were analyzed by electrophoresis on a 2% agarose gel in TBE buffer and stained with ethidium bromide. The expected products were visualized under UV light, excised from the gel and purified with the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The purified PCR products were then used for direct sequencing by First BASE Laboratories SDN BHD (Selangor Darul Ehsan, Malaysia).

Assembly of Genome Sequences and Sequence Analysis

Genome sequences were analyzed using the BLAST programme available in NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Subsequently, they were edited and assembled in CHROMASLITE (v.2.0) and SeqMan (DNASTAR, Madison, WI) software. All sequences were aligned using Clustal X version 1.83 and phylogenetic trees were constructed applying the neighbor-joining method and Kimura's two-parameter with 1,000 bootstrapping method implemented in the MEGA 3.1 program.

RESULTS

Complete genome analysis of CHIKV in Thailand

We analyzed the nearly complete genome sequences of four CHIKV isolates representative of the 2008 and 2009 outbreaks in Thailand (Table 1). Novel amino acid changes detected in Thailand strains as compared to S27 are shown in Table 2. The length of genome sequences of four isolates presented in this paper was 11,811 base pairs except for the isolate CU-Chik661 with a sequence of 11,738 base pairs. Subsequently, they were aligned with 27 complete genome sequences available in GenBank. Overall, the genome structures of these four isolates were consistent with the previous work. The isolates analyzed in this study were very closely related, showing a nucleotide identity of 99.79-99.89% with one another. Furthermore, they showed an average whole genome nucleotide similarity of 97.0% to the S27 prototype, 99.83% to a Malaysian isolate (Accession number FJ807899) and 99.81% to a Singapore isolate (Accession number FJ807896).

Non-structural region

Compared to S27, the CU-Chik661, CU-Chik009, CU-Chik10 and CU-Chik683 isolates displayed 32 identical substitutions in the non-structural region: nine in nsP1 (T128K, L172V, E234K, T376M, M383L, I384L, T481I, Q488R, and L507R), six in nsP2 (S54N, H374Y, C582Y, S582N, L539S and A793V), eleven in nsP3 (V175I, Y217H, P326S, V331A, T337I, K352E, I376T, A382T, L461P, S462N, and P471S) and six in nsP4 (T75A, T254A, Q500L, I514T, V555I, and V604I). These changes were also found in other isolates from the Indian Ocean Islands in 2006 and 2007 (Schuffenecker *et al.*, 2006), Kerala, South India in 2006-2008 (Sreekumar *et al.*, 2009), Sri Lanka, Singapore (Hapuarachchi *et al.*, 2010) and Malaysia (Huang *et al.*, 2009).

Interestingly, at codon 524 of nsP3, there was an opal stop codon (UGA) in the present isolates that was not observed in S27 and Ross strains. This opal stop codon was also detected in related alphaviruses and previously reported CHIKV isolates (Schuffenecker *et al.*, 2006). It is believed to have an important role in regulating the expression of nsP4 by read-through mechanism (Strauss & Strauss, 1994). Additional specific changes were also observed in CU-Chik10 (nsP1-P29S, nsP1-N186D, nsP2-P79S, nsP3-T338M, nsP4-N595K, nsP4-R600I, nsP4-T605S, and nsP4-L606M) and CU-Chik661 (nsP4-Y87H) isolates. There was also a unique nucleotide substitution in the isolates of this

Table 1. Sample details

Sample code	Date of collection	Place	GenBank Acc. No.	Sample type
CU-Chik661	25 May 2009	Narathiwat	GQ905863	Serum
CU-Ckik009*	4 Sep 2009	Bangkok	GU301779	Serum
CU-Ckik10	21 Oct 2008	Narathiwat	GU301780	Serum
CU-Chik683	27 Jul 2009	Narathiwat	GU301781	Culture isolate

* patient returned from Nakhonsrithammaraj, the province in the Southern Thailand.

Table 2. Novel amino acid substitutions identified in Thailand CHIKV isolates

Region	polypeptide position	protein position	S27	CU-Chik661	CU-Chik009	CU-Chik10	CU-Chik683
nsP1	29	29	P	.	.	S	.
	186	186	N	.	.	D	.
nsP2	614	79	P	.	.	S	.
nsP3	1671	338	T	.	.	M	.
nsP4	1950	87	Y	H	.	.	.
	2458	595	N	.	.	K	.
	2463	600	R	.	.	I	.
	2468	605	T	.	.	S	.
	2469	606	L	.	.	M	.
E3	290	30	K	.	R	.	.
E2	409	84	F	.	L	.	.
	632	307	Q	.	R	.	.

study; a synonymous change from A to G at nucleotide position 6811 in the nsP4 region.

Structural region

Analysis of amino acid changes of the structural polyprotein showed 25 positions that were common to all four isolates: three in C (P23S, V27I and K63R), one in E3 (I24T), fifteen in E2 (G57K, I74M, G79E, N160T, A164T, L181M, S194G, I211T, K252Q, M267R, S299M, T312M, A344T, S375T, and V386A), two in 6K (V8I and I54V) and four in E1 (A226V, M269V, D284E, and V322A) proteins. The only isolate displaying specific changes was CU-Chik009 with three unique amino acid changes (E3-K30R, E2-F84L and E2-Q307R). At nucleotide position 9138, isolates of this study had reverted back to wild type nucleotide (T), which had mutated to C in all previously reported recent CHIKV isolates.

5' and 3' UTRs

The 5' UTRs of all four isolates were largely similar to recently described isolates. Only CU-Chik10 showed a mutation (T→A) at nucleotide position 64. Neither insertion nor deletion was observed. Within the 3'UTR, the sequences determined in this study showed a 14-nucleotide (A) deletion in a stretch of 19 nucleotides (A) from positions 11,369 to 11,342 as compared to S27. The same deletion was previously reported in the 2006 Indian Ocean isolates (Schuffenecker *et al.*, 2006). However, CU-Chik661 isolates showed only a single nucleotide (A) deletion in this region.

Phylogenetic analyses

Figure A1 illustrates the phylogenetic tree based on full genome analysis. Our isolates (CU-Chik009, CU-Chik10, CU-Chik683 and CU-Chik661) were most closely related to isolates from Malaysia (Accession No. FJ807899). Moreover, they cluster together with isolates from 2007-2008 Indian, Sri Lankan, Italian and Singapore outbreaks. We also determined the partial *E1* gene sequences as this region has been extensively used to infer the phylogenetic relationships of CHIKV. The phylogenetic tree based on the partial *E1* gene sequence showed all study isolates within the ECSA phylogenetic group (Figure A2). In contrast, the 1958 outbreak in Thailand had been ascribed to a strain of the Asian genotype.

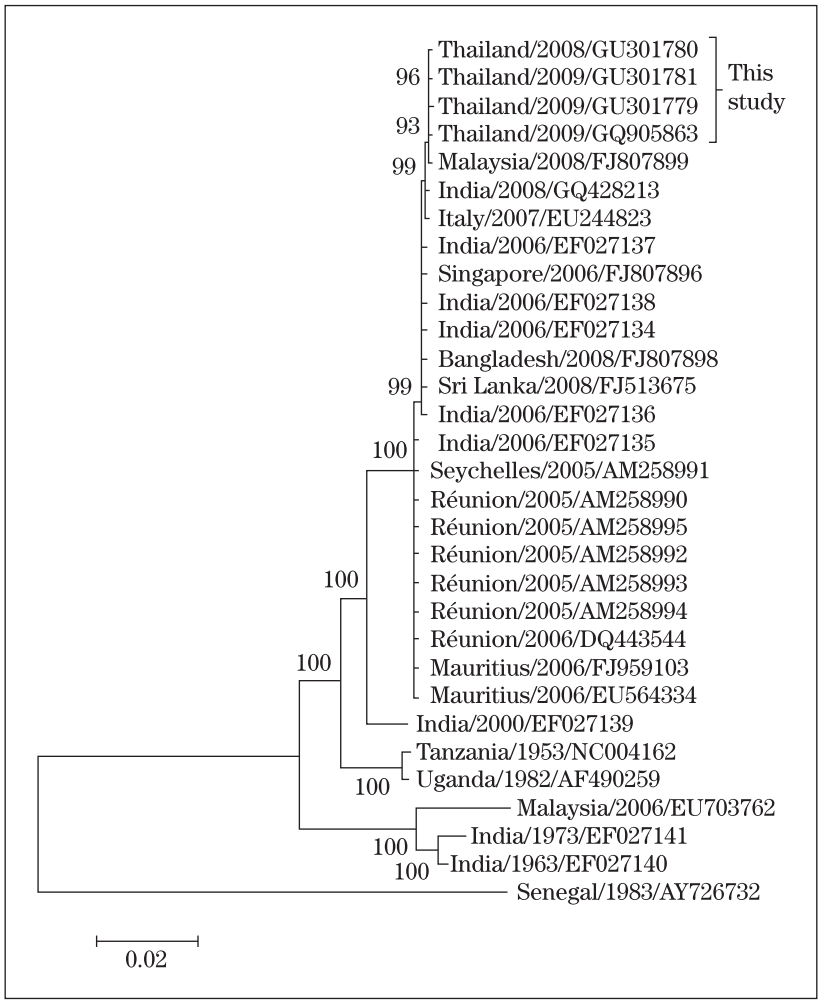

Figure A1. Phylogenetic tree based on the whole genome of CHIKV. Viruses were identified by using the nomenclature of country/year of isolation/ GenBank accession number. Scale bar in the bottom indicates the genetic distance in nucleotide substitutions per site. Numbers represent the bootstrap support obtained for respective branches

DISCUSSION

In Thailand, CHIKV was first described in Bangkok in 1958 (Hammon & Sather, 1964) and was an Asian genotype strain (Powers *et al.*, 2000). Subsequently, CHIKV emerged in Thailand with gaps of 2-18 years between outbreaks: Prachinburi (1976), Surinn (1988), Khon Khen (1991), Loei and Prayao (1993), and Nongkhai & Nakorn Sri Thammaraj (1995). All these outbreaks were caused by CHIKV of the Asian genotype (Powers *et al.*, 2000). CHIKV is presently causing one of the largest outbreaks reported in the past 50 years. Starting in October 2008, a cluster of patients from several villages presenting with fever, rash and severe arthralgia was detected at Laharn health center in Narathiwat. CHIKV was suspected and subsequently confirmed by the HI (Hemaggutination inhibitor) test and RT-PCR methods (Ungchusak, 2008). Since then, CHIKV has been spreading to the adjacent and nearby provinces of Narathiwat including Songkhla, Pattani and

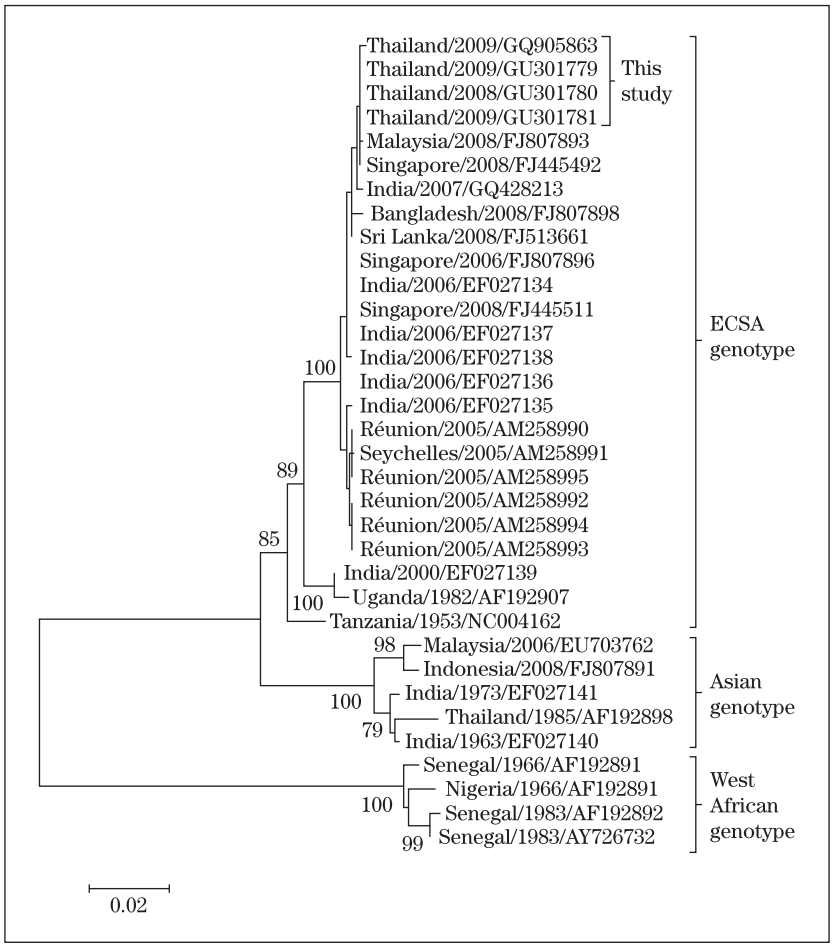

Figure A2. Phylogenetic tree based on the partial E1 gene sequences. Viruses were identified by using the nomenclature of country/year of isolation/ GenBank accession number. Scale bar in the bottom indicates the genetic distance in nucleotide substitutions per site. Numbers represent the bootstrap support obtained for respective branches

Yala with several thousands of cases reported in each area. Not only has CHIKV been disseminated in the nearby area of Narathiwat but is also circulating in other parts of Thailand including the Southeast, Central, North and East regions of the country having caused more than 46,000 infected cases (Bureau of Epidemiology, Department of Disease Control, Thailand, 2008). Due to their magnitude, these epidemics have raised a public health concern in Thailand. Consequently, the Bureau of Epidemiology, Thailand included CHIKF as one of the notifiable diseases and launched a nationwide passive surveillance programme involving all government and some private hospitals in November 2008. Considering the preceding outbreaks in Malaysia, it was assumed that CHIKV might have been introduced from Malaysia into the Southern parts of Thailand where both countries share the border.

This study has demonstrated a high level of similarity among CHIKV isolates detected during the same outbreak. As compared to high levels of genetic drift expected in an RNA genome, this low level of genetic variation suggested a high level of conservation of this RNA virus in the course of a particular outbreak.

Our study represents the first molecular epidemiological analysis, to our knowledge, of a CHIKV outbreak in Thailand. In this study, the phylogenetic analysis based on partial glycoprotein *E1* sequences further confirmed that recent CHIKV dissemination in Thailand was caused by a virus of the ECSA genotype. The same genotype has been identified during recent outbreaks in Malaysia, Singapore, Indonesia, Sri Lanka, Reunion, Seychelles, Mayotte, Madagascar, Mauritius, and India. This is in contrast to the Asian genotype virus strains that were responsible for previous outbreaks in Asia.

Interestingly, all CHIKV isolates analyzed during the current outbreak in Thailand have shown the E1-A226V substitution (Rianthavorn *et al.*, 2010). In Semliki Forest Virus (SFV), which belongs to the same family as CHIKV, protein E1 mediates the entry of the virus into host cells via fusion process, and cholesterol plays an important role in mediating the binding of the viral envelop to the target membranes (Waarts *et al.*, 2002). Previous studies have shown that the E1-A226V mutation of SFV did abolish the cholesterol dependence of the virus (Vashishtha *et al.*, 1998; Ahn *et al.*, 1999). This substitution, therefore, enables the virus to evade cholesterol-independent mosquitoes such as *Ae. albopictus*. Correspondingly, the most predominant mosquito species transmitting the wild-type (E1-A226) CHIKV used to be *Aedes luteocephalus*, *Aedes furcifer*, and *Aedes taylori* in Africa and mostly *Aedes aegypti* in Asia. However, recent reports have demonstrated *Ae. albopictus* as the predominant species in many CHIKF outbreak areas, which coincided with the spread of mutant CHIKV (E1-A226V). In addition, during more recent outbreaks, CHIKV has spread to several regions of the world, where *Ae. albopictus* is existent, but not *Ae. aegypti*, further indicating the potential of *Ae. albopictus* mosquitoes in transmitting the virus (Kumar *et al.*, 2008).

Another interesting observation was the presence of an opal stop codon in nsP3 region. The same observation has been reported in all recently reported CHIKV isolates. It is known that the population of quasispecies of viruses could seep from repeated *in vitro* passages. Therefore, restricting the number of passages is very important because the infecting viral population may keep up a correspondence to a quasispecies (Domingo & Holland, 1997). Accordingly, the appearance of an Arg codon instead of the opal stop codon in S27 is perhaps explained by numerous *in vitro* passages of S27. This is further explained in a previous experiment, which has shown that the Opal stop codon could change to Arg in ONN viruses (Lanciotti *et al.*, 1998).

Though the analyzed CHIKV isolates from Thailand were genetically closest to those described recently in Malaysia and Singapore (Hapuarachchi *et al.*, 2010, Huang *et al.*, 2009), we detected several novel amino acid substitutions in our small cohort of viruses. As the virus is still circulating in certain parts of Thailand, further evolution is expected within the Thailand CHIKV population. This warrants further investigation of a large cohort of CHIKV from different parts of the country, in order to better understand the evolution of CHIKV in Thailand.

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