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An outbreak of chikungunya in southern Thailand from 2008 to 2009 caused by African strains with A226V mutation

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SUMMARY

Objectives: To elucidate clinical and molecular characteristics of chikungunya fever (CHIK fever) from the 2008–2009 outbreak caused by chikungunya virus (CHIKV) in southern Thailand.
Methods: Three hundred and eighty-one sera from 332 patients with acute febrile illness were tested for anti-CHIKV IgM antibody by ELISA. A molecular analysis of these sera was performed using a semi-nested reverse transcriptase polymerase chain reaction (RT-PCR), followed by direct sequencing and phylogenetic analysis.
Results: One hundred and seventy-nine patients were diagnosed with CHIK fever by molecular analysis and/or anti-CHIKV IgM antibody detection. Patients diagnosed with CHIK fever were significantly older than controls (mean age 38.8 ± 19 vs. 28.7 ± 18 years, $p < 0.0001$) and presented with arthralgia more often than controls. One hundred percent of the sera were positive by RT-PCR, whereas only 10% were positive in serological tests for anti-CHIKV IgM antibody by ELISA if the serum was obtained during the first 4 days of fever. In contrast, CHIKV-specific IgM antibody by ELISA was found in 100% of patients, whereas 15% of patients were positive by RT-PCR if the serum was obtained more than 9 days after the onset of fever. RT-PCR for CHIKV should be performed if the patients present within the first 4 days of fever. Patients presenting after at least 9 days of fever should be tested for IgM antibody. Based on phylogenetic analysis, the CHIKV strains isolated belong to African genotypes harboring the E1 A226V mutation, indicating a single origin of the 2004–2009 CHIKV outbreaks.
Conclusions: The novel CHIKV mutation could potentially modify the epidemiological presentation of CHIK fever. Early diagnosis of CHIK fever is essential for preventing further massive outbreaks.

1. Introduction

Since the initial discovery of chikungunya virus (CHIKV) in Tanzania, East Africa in 1952–1953,[1] knowledge on chikungunya fever (CHIK fever) has been continuously evolving. In Asia, the first CHIK fever outbreak occurred in Thailand in 1958.[2] CHIKV circulated in the area until 1962–1964.[3] Several small outbreaks occurred sporadically in different areas, but the last outbreak in Thailand occurred in the mid-1970s. Other CHIK-endemic countries have faced similar situations. CHIKV virtually disappeared for approximately three decades, but more recently has re-emerged in the form of sudden outbreaks in Africa, the Indian Ocean, and Asia.

Relatively little is known about CHIKV despite its discovery more than 50 years ago. The disease has received little attention because it has generally been regarded as self-limiting with few severe complications. This is currently being reconsidered in the light of significant evidence that neurological complications are not as uncommon as was previously believed.[4,5] Upon realizing the severity of recent outbreaks, scientists around the world have raised significant concerns about CHIKV.

The current outbreaks began in Tanzania in 2004 and CHIKV has subsequently been introduced into many countries worldwide as a result of infected humans returning from epidemic areas. CHIKV was isolated on Reunion Island[6] and in India[7] during the 2005–2006 outbreaks. Europe, the USA, and Australia have recently seen sporadic introduced cases of CHIK fever, especially in travelers returning from epidemic regions. Italy experienced an outbreak that affected thousands of people[8] resulting from the virus being introduced by infected international travelers from epidemic areas[9] into the regions where *Aedes albopictus* is present. Unusual

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complications including CHIKV-associated deaths and neurological involvements such as Guillain–Barré syndrome[5] have been reported. Vertical transmission has been observed during these outbreaks.[10]

The recognized vector for CHIK fever is the *Aedes aegypti* mosquito, with *A. albopictus* as the secondary vector. Recently, several countries including Indian Ocean islands,[11] Kerala State in India,[12] Gabon,[13] and Italy[8] have reported *A. albopictus* as a potential vector for CHIKV during recent outbreaks. Moreover, additional molecular virus research performed in the course of these outbreaks has demonstrated a variant of the early CHIKV associated with many of the more recent outbreaks. This variant possesses a mutation from alanine to valine at amino acid position 226 of the E1 envelope glycoprotein gene (E1 A226V mutation).[12,14,15] This mutation enhances the ability of the salivary gland of *A. albopictus* mosquitoes to become infected and thus increases the capability of the mosquitoes to transmit the virus to another host.[15]

In September 2008, the Department of Disease Control of the Thai Ministry of Public Health (MOPH) reported an outbreak of 200 cases diagnosed with CHIK fever in southern Thailand near the border with Malaysia.[16] CHIKV was isolated from *A. albopictus* mosquitoes trapped in the affected area. The outbreak has not yet been controlled. So far, thousands of people have been infected. This study was designed to elucidate the clinical manifestations, molecular characteristics, and vectors of CHIKV in southern Thailand in order to facilitate early diagnosis, control this outbreak, and prevent further outbreaks.

2. Patients and methods

Starting in September 2008, a major CHIK fever outbreak has occurred in southern Thailand near the Malaysian border, mainly affecting Narathiwat Province. Patients who presented at the Narathiwatratchanakharin Provincial Hospital with acute febrile illness were enrolled in this study. The demographic and clinical data of these patients were obtained from medical records. The study was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University for using anonymous specimens for further investigation upon permission by the directors of Chulalongkorn Hospital and Narathiwatratchanakharin Provincial Hospital.

3. Patients

From 332 patients with acute febrile illness, 381 sera were obtained. Baseline clinical data including the history of present illness, physical examination, and complete blood count were obtained by the clinicians at Narathiwatratchanakharin Provincial Hospital. Chikungunya infection was investigated at the Center of Excellence in Clinical Virology, Chulalongkorn University, by serology and molecular virus research. CHIK fever was diagnosed based on amplification of CHIKV RNA by semi-nested reverse transcriptase polymerase chain reaction (RT-PCR) and/or IgM antibody detection. Sera were tested for IgM antibodies to CHIKV by ELISA using commercially available kits (SD BIOLINE, Kyonggido, Korea). Semi-nested RT-PCR was performed to detect CHIKV RNA. The RT-PCR products specific for CHIKV were subjected to direct sequencing.

4. Detection of CHIKV RNA by semi-nested RT-PCR

CHIKV-RNA was extracted from 50 μl of each serum sample by the guanidium isothiocyanate method as described elsewhere,[17] and subsequently reverse-transcribed into cDNA using random hexamer primers. The cDNA was subsequently amplified by semi-nested PCR. For the first PCR, DVRChkF 5′-ACCGGCGTC TACCCATTCATGT-3′ (nt 10237–10258)[18] and CU3-CHIKR 5′-TCGCTRCAGCACACRGCACC-3′ (nt 10741–10760) were used as forward primer and reverse primer, respectively.

The first PCR was performed in a thermocycler model 9600 (Perkin Elmer Cetus, Norwalk, CT, USA). Cycling conditions included initial denaturation at 95 °C for 3 min, followed by 40 amplification cycles of 1 min at 95 °C for denaturation, 1 min at 55 °C for primer annealing, 1 min at 72 °C for extension, and 10 min at 72 °C for final extension. Then, the semi-nested PCR was performed by using CU1CHIKF 5′-GCATCAGCTAAGCTCCGCGTC-3′ (nt 10378–10398) as an inner forward primer. After electrophoresis in a 2% agarose gel stained with ethidium bromide, the expected 532-bp bands were visualized on a UV transilluminator.

5. CHIKV sequencing and genotype characterization

We randomly selected PCR products for sequencing. The PCR products were purified using the Perfectprep Gel Cleanup Kit (Eppendorf, Westbury, NY, USA) according to the manufacturer's specifications and subsequently subjected to 2% agarose gel electrophoresis in order to ascertain their purity.

To determine the concentration of the amplified DNA, each sample was analyzed by absorption at 260 nm in a BioPhotometer (Eppendorf, Hamburg, Germany). The DNA concentration was calculated based on the conversion of 1 OD_{260} being equivalent to 50 g double-stranded DNA. Between 10 and 30 ng/l (3–6 l) of each DNA sample were subjected to sequencing.

6. Phylogenetic analysis of isolated CHIKV sequences

The sequences were determined by BLAST/FASTA (http://www.ncbi.nlm.nih.gov) and phylogenetic analysis. The sequences were edited and assembled using programs CHROMAS LITE v. 2.0 (http://www.technelysium.com.au/chromas_lite.html) and SeqMan (DNASTAR, Madison, WI, USA). To investigate the relationship between CHIKV strains, the un-rooted tree topology based on multiple alignments of the E1 gene nucleotide sequences and those of known wild types and mutants from GenBank was established by the neighbor-joining method calculated with MEGA 3.1 (http://www.megasoftware.net). Consistency of branching was tested by bootstrap analysis with 1000 re-samplings of the data using MEGA 3.1. Multiple protein translation and sequence alignments were generated with BioEdit version 7.0.1 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The sequences of the isolated strains were grouped and compared with the corresponding sequences of CHIKV stored at the GenBank database.

7. Statistical analysis

Variables were summarized including mean and standard deviations (SD). The relationships between gender, symptoms (skin rash and arthralgia), and CHIK fever status (positive and negative) were analyzed by Fisher's exact test. Differences in age, body temperature, hemoglobin, and platelets between CHIK patients and controls were assessed by Student's *t*-test. The statistical analyses were performed with SPSS software version 11 (SPSS Inc., Chicago, IL, USA). *p*-Values below 0.05 (5%) were considered statistically significant.

8. Results

Of the 332 patients enrolled in the study, 179 patients were diagnosed with CHIK fever either based on amplification by semi-nested RT-PCR or IgM antibody by ELISA. Of these 179 patients, 125 (70%) were diagnosed with CHIK fever based on semi-nested RT-PCR

Table 1
Demographic and clinical data of patients with chikungunya fever and controls

	Chikungunya fever (n = 179)	Controls (n = 153)	p-Value
Male:female ratio	78:101	76:77	NS
Age (years) mean ± SD	38.8 ± 19	28.7 ± 18	<0.0001
Rash	6	7	NS
Arthralgia	148	97	<0.0001
Body temperature	38.8 ± 0.8	38.9 ± 0.7	NS
Hemoglobin	13.2 ± 1.7	13.1 ± 1.9	NS
Platelets	209 ± 68	204 ± 113	NS

NS, not significant.

and 40 (22%) were diagnosed based on IgM antibody detection by ELISA. Fourteen patients (8%) proved positive both by semi-nested RT-PCR and IgM antibody in the same serum sample. There were 101 females and 78 males, and age ranged from 2 to 84 years (mean 38.8 years). A predilection by sex was not noted among patients with CHIK fever. Patients with CHIK fever were significantly older than patients diagnosed as negative for CHIK fever (control group) ($p < 0.0001$). Additional symptoms and laboratory results are shown in Table 1. Degree of fever and the frequency of skin rash did not differ between the two groups. However, patients with CHIK fever presented with arthralgia more frequently than the control group and this was statistically significant ($p < 0.0001$). The levels of hemoglobin, and platelet counts did not differ between patients with CHIK fever and the control group.

Further analyses revealed that among the 125 patients with CHIK fever based on semi-nested RT-PCR, 114 (91%) presented during the first 4 days after the onset of fever, whereas only 11 (9%) presented later than 5 days after the onset of fever. IgM antibody for CHIKV was detected in the sera from the first few days after the onset of fever and lasted for at least 35 days.

From 49 of 125 patients diagnosed with CHIK fever based on semi-nested RT-PCR, the second set of sera obtained demonstrated disappearance of CHIKV and/or seroconversion in the second specimen in direct relation to duration of fever, as shown in Figure 1. RT-PCR for CHIKV was 100% positive, whereas the IgM antibody was detected in only 10% of the specimens if the first serum sample was obtained during days 1–4 of fever (Figure 1). The percentage of positive RT-PCR for CHIKV was markedly decreased when the specimens were obtained from day 5 onwards following the onset of fever. By days 9–12 of fever, all patients were positive for IgM antibody against CHIKV, whereas only 15% of patients were positive by RT-PCR.

9. Molecular virus characterization

All isolated CHIKV strains that were randomly selected for sequencing were submitted to the GenBank database under accession numbers **FJ882857**–**FJ882922**. All CHIKV strains from this outbreak were closely related to the African genotypes and to strains isolated from the 2007 outbreaks in India (99–100%), as shown in Figure 2, implying a common origin.[12] The nucleotide sequence homology shared among all CHIKV strains isolated in this study was more than 99%. Moreover, all displayed the E1 A226V mutation. However, the homology between the sequences of strains isolated from this study and those from the previous outbreaks in Thailand (1962–1996) was only 92–93%, suggesting that CHIKV was imported to Thailand and caused the 2008 outbreak.

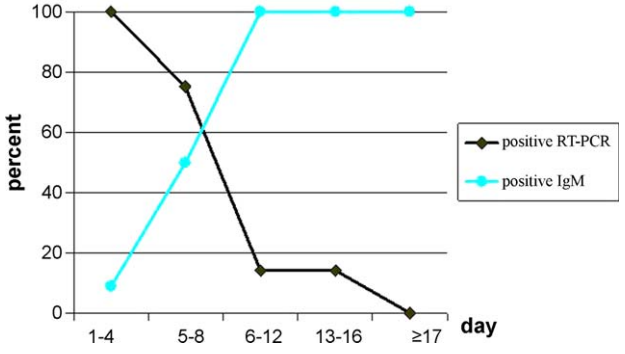

Figure 1. Percent of paired sera specimens with chikungunya virus (CHIKV) positive by semi-nested RT-PCR or anti-CHIKV IgM in relation to duration of fever.

10. Discussion

Based on the results of this study, the only significant sign for CHIK fever was arthralgia, consistent with the name coined in 1955 'chikungunya', which means 'that which contorts or bends up'.[1,19] However, the degree of fever and the incidence of skin rash did not differ between patients with CHIK fever and the control group. No predilection by sex was observed among patients with CHIK fever. Unlike in dengue hemorrhagic fever, patients with CHIK fever did not develop significant hemoconcentration or thrombocytopenia. After the first diagnosis and outbreak of CHIK in Thailand in 1958,[1] an estimated 40 000 people were infected during the 1960 s due to heavy infestation by *A. aegypti* in the water containers ubiquitous in Thai households.[2] CHIKV transmission nearly disappeared from Thailand during the mid 1970 s despite the continued presence of the presumed vectors in the area. This observation is supported by the report of Burke et al.,[20] that lack of exposure to CHIKV after the 1970 s led to low baseline protective immunity and thus, rendered the population susceptible to the virus. Hence, antibodies to CHIKV were rare in Bangkok children born after 1976. Several small sporadic outbreaks were reported from Khon Kaen (July 1991), Nakorn Si Thammarat (July 1995), and Nong Khai (August 1995) provinces in the 1990s.[21] However, the virus re-emerged as a major epidemic in 2008. The reasons for the disappearance of CHIKV, despite the presence of the appropriate vector, have not been adequately explained, although it seems likely that suitable reservoir hosts other than humans may not have been present during the period of apparent 'silence'.

Demographically, the mean age of patients presenting with CHIK fever in this study was approximately 40 years, suggesting that adults had rarely been exposed to CHIKV before and did not have protective antibodies. Watanaveeradej et al.[22] reported high seroprevalence of antibodies to CHIKV by hemagglutination inhibition of up to 45% in Thai pregnant women over the age of 35 years, although this percentage positivity in this particular group of women does not ensure that herd immunity is sufficient to protect the population against an incoming strain of virus. Thus, the Thai population may still be susceptible to CHIKV. Similarly, a report from Sri Lanka, another CHIK fever endemic country, demonstrated no evidence of antibodies against CHIKV before the most recent outbreak, making the population susceptible to the virus.[23]

Serology and molecular virus research are rapid and accurate methods for diagnosis of CHIK fever. During the first 4 days of fever, viremia was detected by semi-nested RT-PCR in 100% of patients, whereas IgM antibody was detected in approximately 10% of patients. The chance of having viremia decreased as the IgM antibody developed. During days 9–12 of fever, the IgM antibody developed in all patients, while CHIKV was still detectable by the semi-nested RT-PCR in approximately 15% of patients.

CHIKV has been known to be transmitted by *A. aegypti* mosquitoes. Recent in vitro experimental studies have shown that *A. albopictus* is essentially an important vector for CHIKV.[15,24] CHIKV has been isolated from *A. albopictus* mosquitoes trapped during

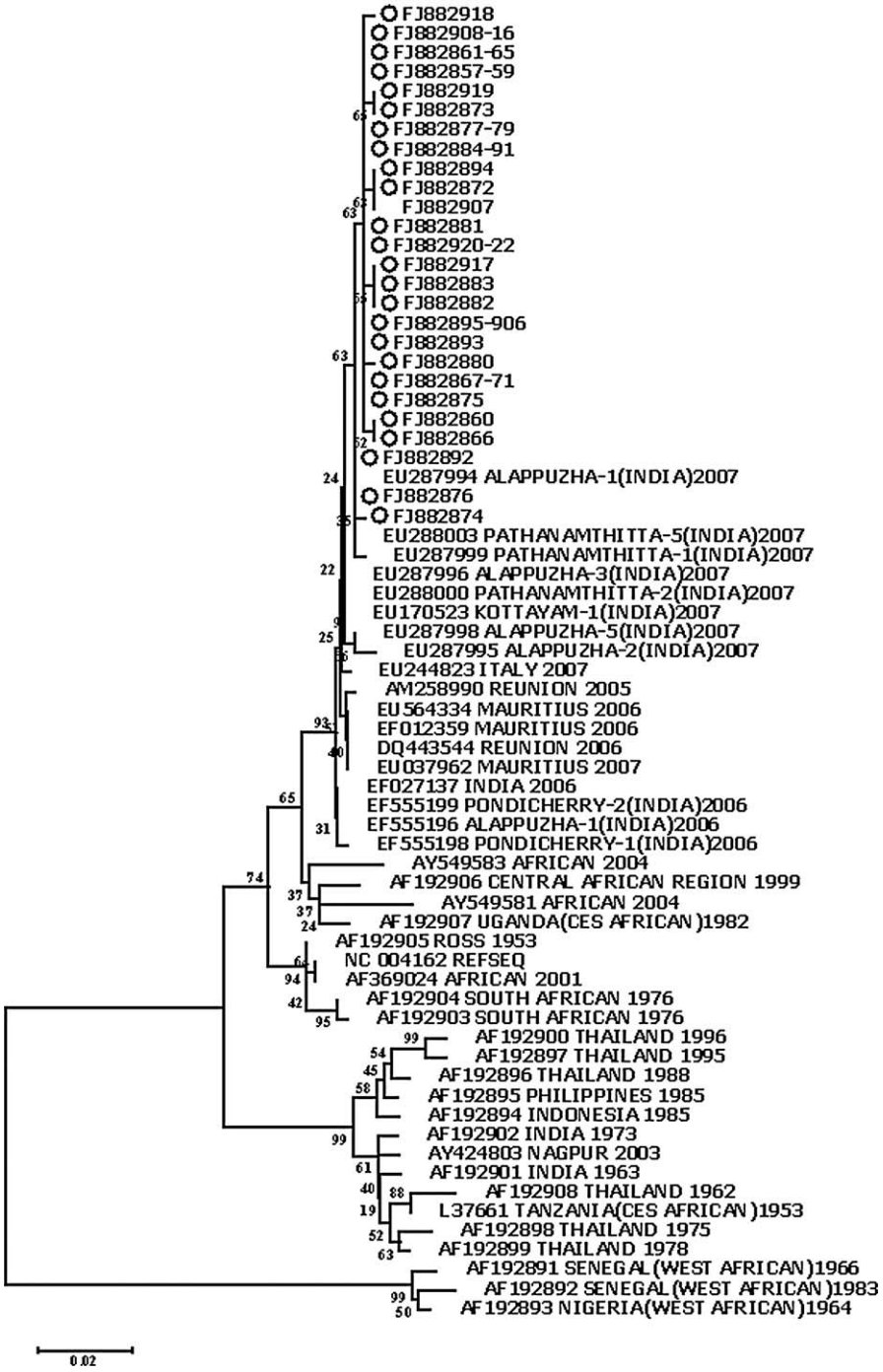

Figure 2. Phylogenetic tree showing the relationship between chikungunya virus (CHIKV) strains using un-rooted tree topology based on multiple alignments of the E1 gene nucleotide sequences and those of known wild types and mutants from GenBank, established by the neighbor-joining method calculated with MEGA 3.1. Open circles represent CHIKV strains isolated from this study.

several outbreaks worldwide,[11,13,16] including this outbreak in southern Thailand.[25] According to the study presented here, CHIKV isolated from this outbreak harbors the new mutation (A226V) in the E1 envelope glycoprotein gene similar to that found in those isolated during the recent outbreaks in other countries.[26] This mutation significantly increases CHIKV transmissibility to and by *A. albopictus* mosquitoes, which provides a selective advantage over infection in *A. aegypti*.[10,23] In Thailand, *A. albopictus* is abundant in the para rubber tree (*Hevea brasiliensis*) plantation area, where people residing in southern Thailand work as rubber tappers. Thus, vector control is evidently one of the most essential means to reduce the risk of further outbreaks of CHIKV in southern Thailand.

The novel CHIKV mutation and worldwide dispersal of *A. albopictus* mosquitoes[9,11–13,25] could potentially modify the epidemiological presentation of CHIK fever. According to the study presented here, the isolated CHIKV strains harbor the E1 A226V mutation. Moreover, *A. albopictus* is not only found in tropical regions; this species is also now becoming established in more temperate climates. Thus, as suggested previously,[15,25,26] CHIKV has the potential to present a threat to public health even in developed countries. Healthcare providers should be made aware that in tropical regions and potentially in temperate regions where *A. albopictus* has become established, CHIKV could present a serious threat. A state of awareness should be communicated to

the relevant medical personnel in hospitals, especially when patients present with high-grade fever accompanied by severe arthralgia. Confirmation by specific diagnosis is relatively straightforward. All commercial traffic entering the country should be screened for the presence of all stages of *A. albopictus*. Implementation of this measure requires a highly organized infrastructure with well-trained personnel.

11. Conflict of interest

No conflict of interest to declare.

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