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Interepidemic Detection of Chikungunya Virus Infection and Transmission in Northeastern Thailand

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Abstract. Chikungunya fever is a viral mosquito-borne, acute febrile illness associated with rash, joint pain, and occasionally prolonged polyarthritis. Chikungunya outbreaks have been reported worldwide including many provinces of Thailand. Although chikungunya virus (CHIKV) occurs in Thailand, details on its epidemiology are lacking compared with dengue, a common mosquito-borne disease in the country. Therefore, study on CHIKV and its epidemiology in both humans and mosquitoes is required to better understand its importance clinically and dynamics in community settings. So a prospective examination of virus circulation in human and mosquito populations in northeastern Thailand using serological and molecular methods, including the genetic characterization of the virus, was undertaken. The study was conducted among febrile patients in eight district hospitals in northeastern Thailand from June 2016 to October 2017. Using real-time PCR on the conserved region of nonstructural protein 1 gene, CHIKV was detected in eight (4.9%) of 161 plasma samples. Only one strain yielded a sequence of sufficient size allowing for phylogenetic analysis. In addition, anti-CHIKV IgM and IgG were detected in six (3.7%) and 17 (10.6%) patient plasma samples. The single sequenced sample belonged to the East/Central/South Africa (ECSA) genotype and was phylogenetically similar to the Indian Ocean sublineage. Adult *Aedes* mosquitoes were collected indoors and within a 100-m radius from the index case house and four neighboring houses. CHIKV was detected in two of 70 (2.9%) female *Aedes aegypti* mosquito pools. This study clearly demonstrated the presence and local transmission of the ECSA genotype of CHIKV in the northeastern region of Thailand.

INTRODUCTION

Chikungunya fever is typically a self-limiting viral illness caused by chikungunya virus (CHIKV) infection transmitted by specific *Aedes* mosquitoes.[1] The name "chikungunya" originates from the Makonde language in southern Tanzania, translated as "that which bends up," referring to the general posture of an acutely ill patient caused by extreme joint pain and occasionally followed by a prolonged polyarthritis.[2] Chikungunya virus is classified as an *Alphavirus* (formerly Group A arbovirus) in the family *Togaviridae*. CHIKV is a positive-sense, single-stranded RNA virus of about 11.8 kb and currently classified into three main genotypes reflecting the initial geographic isolation of each: West Africa, East/Central/South Africa (ECSA), and Asian.[1] Besides, the Indian Ocean lineage (IOL) of ECSA is now recognized as a fourth variant causing human disease.[3]

After having been identified as the cause of acute febrile outbreaks in Tanzania in 1955, CHIKV infection has been detected in many countries and has more recently become a significant global public health problem.[4,5] The more recent rapid expansion outside Africa and tropical Asia can be attributed to many factors, including increased volume of regional and international travelers, expanding distribution of virus-competent *Aedes* vector mosquitoes, and the adaptation of virus with a global expansion of *Aedes albopictus* (Skuse) mosquitoes outside Asia.[6] Mammals and mosquitoes play essential roles in the epidemiology of CHIKV in which humans and wild primates act as the primary vertebrate hosts, whereas various mosquitoes—primarily *Aedes* species in the subgenera *Stegomyia* and *Diceromyia*—are responsible for transmission.[7,8] Combining correct environmental conditions and susceptible vertebrate host availability with proficient and vectorial capacity and viral genetic diversity has promoted the emergence and reemergence of CHIKV.[9] This is illustrated by recent multicountry epidemics in the Indian Ocean region involving the CHIKV IOL strain with an apparent heightened viral fitness and transmission efficiency in *Ae. albopictus*.[9,10]

Clinically, only 3,504 CHIKV cases have been reported in the Asia–Pacific region between 1954 and 2017[11]; assuredly, this is far below the actual number of symptomatic and subclinical infections occurring during this period. More recently, an increasing number of imported CHIKV infections in travelers returning to non-endemic regions in Europe and North America have been reported.[12] A retrospective molecular and seroepidemiological study showed CHIKV is widely distributed in many countries throughout South, Southeast Asia, and Pacific regions.[13] In Southeast Asia, chikungunya-like fever has been reported since the late 1700s with the first isolation in Thailand in 1958 as a coinfection with dengue virus (DENV).[11] Subsequently, the Asian genotype of CHIKV has been detected in many areas in the region since the late 1950s and

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continues to produce sporadic outbreaks.[14] Currently, the IOL is the most active lineage reported in Southeast Asia, belonging to the ECSA genotype with a distinctive envelope E1 gene mutation amino acid substitution (alanine to valine) at the 226 nucleotide position.[15] Outbreaks of CHIKV in Malaysia,[15,16] Singapore.[17] and earlier in the Philippines[14,15] have posed challenges for investigation and control. Together with dengue and Zika virus surveillance, investigations on CHIKV are now possible in many countries in Southeast Asia (e.g., the Philippines, Vietnam, Myanmar, Lao PDR, and Cambodia), which will provide a better analysis of the distribution of CHIKV infection in the region.[14,18–22]

Thailand, particularly urban Bangkok, is a remarkably active location for CHIKV infection and transmission.[23] Historically, from the first reported outbreak in 1958,[24] the virus was identified in other areas up until 1964,[25] then reemerged in 1975 and 1976,[23] and again in 2008–2009 in the southern provinces of Thailand, totaling in this entire span of time 49,069 recorded cases.[26] The virus implicated in 2008–2009 outbreaks was ECSA-IOL with *Ae. albopictus* as the primary vector in southern Thailand.[27–29] Interestingly, the CHIKV strains isolated in Narathiwat Province in southern Thailand in 2008 displayed different sequences from those in previous outbreaks[30] but similar to isolates reported from Singapore.[31] In 2010, two mutations of the ECSA genotype, E1-A226V and E2-I211T, were described in patients in central Thailand.[32,33] More recently, importation of CHIKV was reported in travelers returning to their countries of origin (Europe and the Middle East) having acquired the infection from tourist areas in Thailand.[34,35] In northeastern Thailand, outbreaks have been recorded in Khon Kaen (July 1991), Loei and Phayao (1993), and Nong Khai (August 1995) provinces.[36] In 2013, a CHIKV-ECSA outbreak occurred in Bueng Kan Province that borders Lao PDR.[37] Another study examined long-term immunity against CHIKV in human populations in Khon Kaen Province.[38]

Although the circulation of CHIKV in humans and mosquitoes has been documented in many provinces of Thailand,[39–41] genotypic identification of virus circulation in the northeastern region remains limited. Therefore, the objective of this study was to investigate the circulation of CHIKV in human populations and mosquitoes in northeastern Thailand using a combination of serological and molecular detection techniques. A secondary goal was to describe phylogenetically CHIKV strains acquired from acute febrile patient samples.

MATERIALS AND METHODS

Human study population, recruitment, and blood sample collection. An observational study was carried out in four provinces in northeastern Thailand (Khon Kaen, Roi Et, Kalasin, and Maha Sarakham) from June 2016 to October 2017. The study sampling and data collection process is presented in Figure 1. Blood samples were taken after obtaining informed consent from each volunteer patient presenting with acute febrile illness at any one of eight participating district hospitals in a prospective hospital-based dengue case–control study. Hospitals were selected based on historical reporting of high dengue cases, relatively large patient catchment areas, and the willingness of hospital management and staff to participate.

For the case–control study, eligible patients were at least 5 years of age and all initially presenting with uncomplicated fever (> 38°C). For the CHIKV study, cases were drawn from those patients with suspected dengue, with acute febrile illness onset 2–7 days before sampling, and at least two of the following signs/symptoms: persistent headache; retro-orbital pain; muscle, bone, and/or joint pain; rash or flushed face; petechiae; or a positive tourniquet test. Patients younger than 5 years, those with primary residence outside the hospital's catchment area or having been away from their primary residence during the last 7 days, and those patients with severe signs including shock, brain injury, liver failure, or in an unconsciousness state were excluded. Patients with chronic

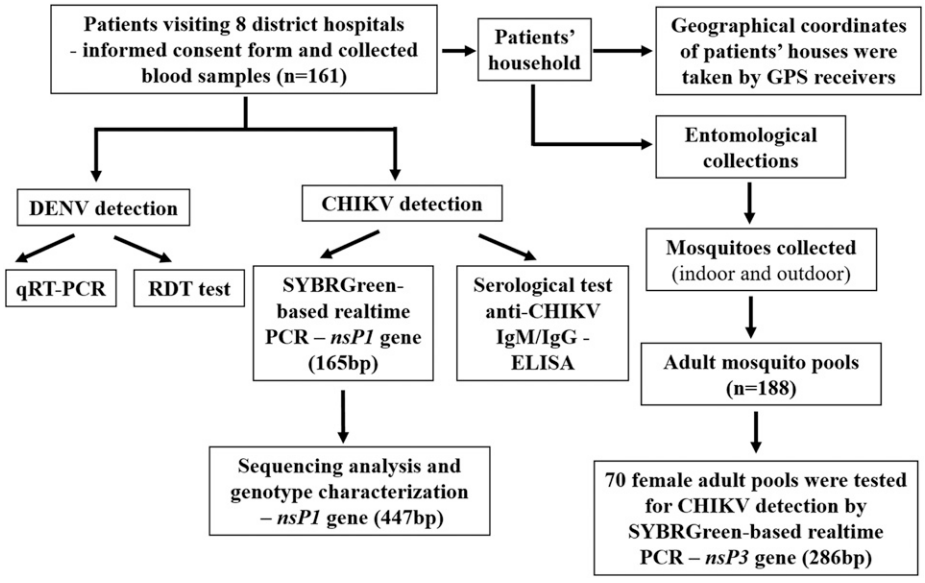

FIGURE 1. Study flowchart. Plasma samples collected from eight district hospitals in Khon Kaen, Roi Et, Maha Sarakham, and Kalasin provinces from June 2016 to October 2017. CHIKV = chikungunya virus; DENV = dengue virus; *nsP1* = nonstructural protein 1; *nsP3* = nonstructural protein 3; RDT = rapid detection test.

fever due to other infections such as HIV or malaria were excluded. After providing signed consent to participate, a single venous blood sample (approximately 10 mL) was collected by using a syringe vacuum tube then divided into separate tubes, one containing heparin (~6 mL blood) and the other ethylenediaminetetraacetic acid (~2 mL blood) (Greiner Bio-One Thailand Ltd, Chonburi, Thailand). The blood tube was transferred to the Department of Microbiology, Khon Kaen University, within 6 hours and immediately subjected to 10-minute centrifugation at 1,300 × *g* in the laboratory. The plasma layer was collected and 140 µL retained for RNA extraction. The remaining plasma was stored at –80°C for other study assays.[42]

The patient's primary home address was geo-referenced using GPS receivers. ArcGIS v10.5.1 (ESRI, Redlands, CA) was used to visualize the geographical coordinates of each patient's house. The standardized classification of patients' age into groups followed a published guideline document.[43]

Case definition. A human chikungunya case was defined as any febrile patient with a positive quantitative reverse transcription polymerase chain reaction (qRT-PCR) and/or evidence of anti-CHIKV immunoglobulins by serological methods. Patients with no reactive test findings were considered "negative" for CHIKV infection. Patients with initial positive findings by using the rapid detection test (RDT) or qRT-PCR for DENV were reported as DENV-infected.

Serological assays. Human plasma samples were tested for semiquantitative determination of human anti-CHIKV antibodies (IgM and IgG) using an ELISA (Euroimmun, Lübeck, Germany). Samples were assayed in 96-well microplates according to the manufacturer's instructions. In brief, plasma samples were diluted 1:101 with provided buffer and added to the microplates containing CHIKV recombinant structural protein. After incubation for 60 minutes at 37°C, plates were washed three times, followed by an incubation step with peroxidase-labeled antihuman IgM/IgG for 30 minutes. After three washing steps, the substrate solution was added and incubated for 15 minutes. After terminating the reaction with stop solution, plates were read within 30 minutes using a spectrophotometer at 450 nm with a 620-nm reference wavelength. The IgM/IgG extinction value is the ratio of the extinction value of the patient sample over the calibrator value following the manufacturer's instructions. Samples were designated as positive for CHIKV-specific immunoglobulin with values above 1.1 and as negative at 0.8 or lower. Those samples with values between 0.8 and 1.1 were borderline (indeterminate) and recorded as negative.

In addition, an RDT test (SD BIOLINE Dengue Duo, Standard Diagnostics, Seoul, Korea) was performed using 100 µL plasma to detect dengue nonstructural protein 1 (nsP1) and separately 10 µL plasma for DENV IgM/IgG antibody. The RDT is not capable of distinguishing between dengue serotypes. Complete dengue results will be published independently.

Molecular assays: viral RNA detection in humans. The genomic RNA was extracted from 140 µL plasma by using a QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Afterward, total RNA was reverse-transcribed into cDNA using 1 µL random hexamer primers and the SuperScript™ III First-Strand Synthesis System (Invitrogen, Waltham, MA) following product instructions. The cDNA was stored at –20°C until further analysis. Screening for CHIKV infection from human plasma was carried out using self-designed *nsP1* gene using SYBR green-based real-time PCR. Sets of primer pairs (Table 1) were designed from the conserved region of the *nsP1* gene and then screened for primer efficiency, specificity, and sensitivity by standard curve methods using 10-fold serial dilutions of plasmid pGEM-T (Promega, Madison, WI) containing target gene CHIKV *nsP1* ranging from 1×10^1 to 10^{11} copies/µL (data not shown). All real-time PCR assays were performed on a CFX-96® Real-Time Detection System (Bio-Rad Laboratories) and analyzed with CFX Manager software (Bio-Rad Laboratories, Hercules, CA). The results were analyzed using the threshold cycle (Ct) value for the amplification plots and melting temperature (Tm) value for verifying specificities of each amplicon. A quantitative, one-step SYBR green-based reverse transcript PCR was performed to differentiate between the four DENV serotypes.[44]

Adult mosquito resting collections at households. Mosquito collections took place at each patient's household and an additional four neighboring houses within a 100-m radius from the index case. Adult daytime resting mosquitoes were collected using portable Prokopack aspirators[45] for 15-minute indoors (primarily in living rooms and bedrooms) and 15-minute outdoors near the house (mainly around human-made articles, vegetation, etc.). Adult mosquitoes from the patient and respective neighboring houses were recorded as one combined collection cluster. For subsequent virological testing, mosquitoes were first separated into pools according to collection cluster, sex (male/female), and species (*Aedes aegypti* or *Ae. albopictus*) using a stereomicroscope to aid in the species identification.[46] Mosquitoes were stored individually in 1.5-mL Eppendorf® tubes (Eppendorf AG, Hamburg, Germany) separated by cotton wool placed over silica

TABLE 1
Primer sets used for PCR amplification

Primer	Sequence (5′–3′)	Target gene	Amplicon size (bp)	Reference
Primers for CHIKV detection from human plasma samples and mosquito pools				
CHIK-nsP1-F	GTGCGTACCCCATGTTTG (118–135)	*nsP1*	165	This study
CHIK-nsP1-R	CCGACATCATCCTCCTTG (282–265)			
CHIK-F	CGAGATACTGCCCGTCCCGT (5128–5147)	*nsP3*	286	Chen et al.[47]
CHIK-R	GTCACGCGTCTCCGCTGTTT (5413–5394)			
Primers for partial sequence CHIKV genes for sequencing and phylogenetic analysis				
CHIK-nsP1-F	GTGCGTACCCCATGTTTG (118–135)	*nsP1*	447	Modified from Hasebe et al[48]
CHIK-nsP1-C	GTGCGTACCCCATGTTTG (579–560)			

CHIKV = chikungunya virus; nsP1 = nonstructural protein 1; nsP3 = nonstructural protein 3.

gel (beads), transported to Khon Kaen University, and stored at –80°C until further processing.

Virus detection in mosquitoes. A total of 188 *Aedes* mosquito pools were processed for RNA extraction. Of those, 70 pools (37.2%) of female adult mosquitoes were tested for CHIKV infection. Abdomens were separated from the head–thorax with the latter section stored at –20°C for further analysis. Whole abdomens, combined with respective mosquito pools, were homogenized in 500 µL of Leibovitz's L-15 medium (Gibco, Thermo Fisher Scientific, Waltham, MA). The homogenized solution was clarified by centrifugation 800 × *g* at 4°C for 5 minutes. RNA from pooled mosquitoes was extracted by using a QIAamp viral RNA mini kit (Qiagen) following the product instructions. Chikungunya virus infection was detected using *nsP3* gene–specific primers and SYBR green-based real-time PCR assay described by Chen.[47] PCR amplification was performed on the Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System (Thermo Fisher Scientific). Results were analyzed following the manufacturer's instructions for the Ct value of amplification plots and Tm value when verifying the specificities of the amplicon.

Partial *nsP1* gene amplification and sequencing. The amplification of the partial *nsP1* gene (fragment of 447 bp) was performed using a modified conventional PCR protocol, in which we used one primer described by Hasebe et al.[48] and combined one self-designed primer to attempt obtaining a larger fragment (447 bp) (Table 1). The PCR reaction was carried out using 1.25 U of high-fidelity PrimeSTAR GXL DNA polymerase (Takara Bio, Mountain View, CA), 0.25 mM dNTPs, 5X PrimeSTAR GXL Buffer (+$MgCl_2$) and 10 µM of each forward and reverse primer, and 4 µL of cDNA in 25 µL reactions. PCR was performed using optimal conditions for ≤ 10 kb products in three steps: an initial 5-minute denaturation at 98°C, followed by 35 cycles at 98°C for 10 seconds, 62°C for 15 seconds, and 68°C for 1 minute. A conventional PCR was performed using a Bio-Rad C1000 thermal cycler (Bio-Rad). Five microliters of PCR product were subjected to 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized with an ultraviolet gel doc transilluminator (Bio-Rad). Samples that produced clear and good density amplicons on PCR were chosen for gel purification using a GF-1 AmbiClean Kit (PCR & Gel) (Vivantis Technologies, Selangor Darul Ehsan, Malaysia) and outsourced for nucleotide sequencing (Solgent Sequencing, Biogenomed, Korea). The nucleotide sequence was subsequently viewed and edited by Free BioEdit software 7.2 (bis biosciences, Carlsbad, CA, Available at: http://www.mbio.ncsu.edu/BioEdit/bioedit.html), then compared with published GenBank reference sequences (https://www.ncbi.nlm.nih.gov/genbank/) using basic local alignment search tool analysis.[49]

Genotyping and phylogenetic analysis. MEGA X software (Institute of Molecular Evolutionary Genetics, The Pennsylvania State University, University Park, PA) was used for phylogenetic analysis.[50] The viral sequences were aligned using ClustalW software embedded in MEGA X. The phylogenetic tree was constructed using the neighbor-joining method along with a bootstrap test with 1,000 replicates for evaluating analytic reliability.[51] Thirty-six sequences (Supplemental Table 1) from known CHIKV lineages (West Africa, ECSA, IOL, and Asian) and one outgroup (O'nyong-nyong virus) were used for building the tree.

Statistical analysis. Patient demographic data by age, gender, and district hospital were analyzed descriptively for confirmed CHIKV exposure, defined by detection using either CHIKV real-time PCR or positive CHIKV-specific IgM/IgG antibodies. Data were analyzed using SPSS Statistics v19 (IBM Corp, Armonk, NY). Geo-referenced maps showing the location (by district) of acute CHIKV positive samples by RT-PCR and IgM were created using ArcGIS v10.5.1 software (ESRI, Redlands, CA).

Ethics review and approval. Archived human samples from a dengue case–control study were used in this investigation.[42] Both studies were approved by the Khon Kaen University Ethics Committee for Human Research (Nos. HE591099 and HE611424) based on the Declaration of Helsinki and the International Conference on Harmonization Good Clinical Practice Guideline.

RESULTS

Human CHIKV infections by serological and molecular detection methods. From June 2016 to October 2017, 161 individual acute febrile patient plasma samples were collected from eight district hospitals in northeastern Thailand. The most common sign was fever, and the mean number of days from symptomatic onset to the presentation at hospital was 3.5 days. Males represented 53.4% (86/161) of detected cases, largely being IgG positive. Among 161 patients, 106 were < 15 years of age and 55 were ≥ 15. Thirty-one patients were identified as having had recent or current CHIKV infection. Chikungunya virus infection was indicated in six (3.7%) patients by anti-CHIKV IgM, 17 (10.6%) by anti-CHIKV IgG, and 8 (4.9%) by qRT-PCR. One patient was positive by qRT-PCR and anti-CHIKV IgG only. Evidence of current or prior CHIKV infection was positively associated with age: 14/55 (25.5%) in those aged ≥ 15 years versus 17/106 (16%) in those aged < 15 years. The highest proportion of anti-IgG (41.4%), indicating a previous infection or possibly the latter phase of a more recent CHIKV infection, was in the 24–64 years -age-group. The highest proportion of CHIKV infections was found in Manchakiri Hospital, reporting 12.5% anti-CHIKV IgM and 25% anti-CHIKV IgG from 16 collected plasma samples. Chiang Yuen Hospital had the highest frequency of confirmed acute CHIKV infection by qRT-PCR with five (29.4%) positive samples from 17 suspected cases (Table 2).

Active circulation of CHIKV infections was indicated in samples with anti-CHIKV IgM and/or qRT-PCR–positive samples. Five cases with anti-CHIKV IgM were seen in 2016 and one case in 2017. Chikungunya virus infections based on qRT-PCR detection comprised two cases in 2016 and six in 2017. Active circulating CHIKV among provinces appeared more in Khon Kaen and Roi Et in 2016 and conversely, Maha Sarakham and Kalasin in 2017 (Figure 2).

Entomological investigations. *Aedes aegypti* was the predominant species (97.8%) in all collection households, with the infrequent occurrence of *Ae. albopictus*. The number of female mosquitoes (*n* = 316, 312 *Ae. aegypti*, 4 *Ae. albopictus*) per pool was between one and 15 individuals. Two of the 70 mosquito pools (2.9%), both *Ae. aegypti*, were found positive for CHIKV by qRT-PCR. No attempt was made to sequence the virus from the mosquito pools. Details on entomological collections are published elsewhere.

Co-circulation of CHIKV and DENV infection in northeastern Thailand. Nine of 60 seropositive DENV patients (15%) were positive by both DENV RDT and anti-CHIKV IgM/IgG ELISA. Two patients had evidence of concurrent coinfection with CHIKV based on qRT-PCR (Table 3).

TABLE 2
Demographic characteristics of human CHIKV infections detected by either serological and/or molecular methods (n = 161)

Variable		Total	Anti-CHIKV IgM	Anti-CHIKV IgG	CHIKV RNA
		% (n)	% Positive (n)	% Positive (n)	% Positive (n)
Gender	Male	53.4 (86)	0 (0)	10.5 (9)	4.7 (4)
	Female	46.6 (75)	8.0 (6)	10.7 (8)	5.3 (4)
Age range (years)	0–14	65.8 (106)	5.7 (6)	3.8 (4)	6.6 (7)
	15–24	15.5 (25)	0 (0)	0 (0)	0 (0)
	24–64	18 (29)	0 (0)	41.4 (12)	3.4 (1)
	≥ 65	0.6 (1)	0 (0)	100 (1)	0 (0)
District hospital	Baan Phai	2.5 (4)	0 (0)	25 (1)	0 (0)
	Chum Phae	22.4 (36)	0 (0)	5.6 (2)	2.8 (1)
	Chiang Yuen	10.6 (17)	5.9 (1)	17.6 (3)	29.4 (5)
	Kutchinarai	5.0 (8)	0 (0)	0 (0)	12.5 (1)
	Mancha Khiri	9.9 (16)	12.5 (2)	25 (4)	0 (0)
	Phon Thong	28.0 (45)	0 (0)	8.9 (4)	0 (0)
	Selaphum	10.6 (17)	11.8 (2)	0 (0)	0 (0)
	Sirindhorn	11.2 (18)	5.6 (1)	16.7 (3)	5.6 (1)
% (Total)		100 (161)	3.7 (6)	10.6 (17)	4.9 (8)

CHIKV = chikungunya virus.

Genetic characteristics of CHIKV strains from humans. Because of low viral load, amplification of the partial *nsP1* gene was possible for only three of the eight CHIKV plasma samples. From these three PCR products, only one sequence was sufficient for alignment with MEGA X software. This single strain, designated as THAI/KhonKaen/CP4005-8/2016 (GenBank accession number MT185940), indicates the patient's district of residence and year of collection. This strain was compared with six West African, 15 Asian, and 15 ECSA viral strains, including a relatively recent ECSA IOL subtype, showing 89.3%, 96.7%, and 97.5% similarity, respectively. Based on the constructed phylogeny, the Chum Phae district isolate represents the ECSA genotype (Figure 3). Moreover, this strain was more closely related to IOL strains isolated in 2005 during outbreaks in Mauritius and La Reunion islands. This finding is also in accordance with a recent study in Chum Phae, Khon Kaen Province.[38]

DISCUSSION

Although there have been previous reports on CHIKV outbreaks in Thailand,[29,31–34,36,37,41] including the northeastern region,[37,38] most investigations have focused on serological screening of populations for evidence of infection (past or current). This study is the first investigation of the presence of CHIKV-infected patients during an interepidemic period of CHIKV and DENV activity based on local Department of Health reporting mechanisms. Eight (4.9%) and 23 (14.3%) of the sampled human population (n = 161) were CHIKV positive by qRT-PCR and anti-CHIKV immunoglobulins (IgM/IgG), respectively. By comparison, Sasayama et al.[32] examined 50 serum samples of suspected chikungunya fever cases from Ratchaburi Province (central Thailand) collected between August and September 2010 and found 12% of samples positive using PCR. In the 2012–2013 outbreak in Champasak Province, southern Lao PDR, 6.8% and 6% of the assayed samples had evidence of either current or previous CHIKV infections using qRT-PCR and serological detection, respectively.[20] Retrospective evidence of CHIKV infection in southern and central Vietnam indicated 13.4% seropositivity from 546 samples.[52] In this study, all CHIKV patients detected by qRT-PCR or serology were obtained from individuals in an acute phase (fever) of illness (≤ 7 days post-onset). Presentation of illness in these patients is presumed to have a CHIKV etiology, except for the two patients identified with CHIKV + DENV coinfection, whereby the specific cause of the febrile illness is unknown. One qRT-PCR positive patient

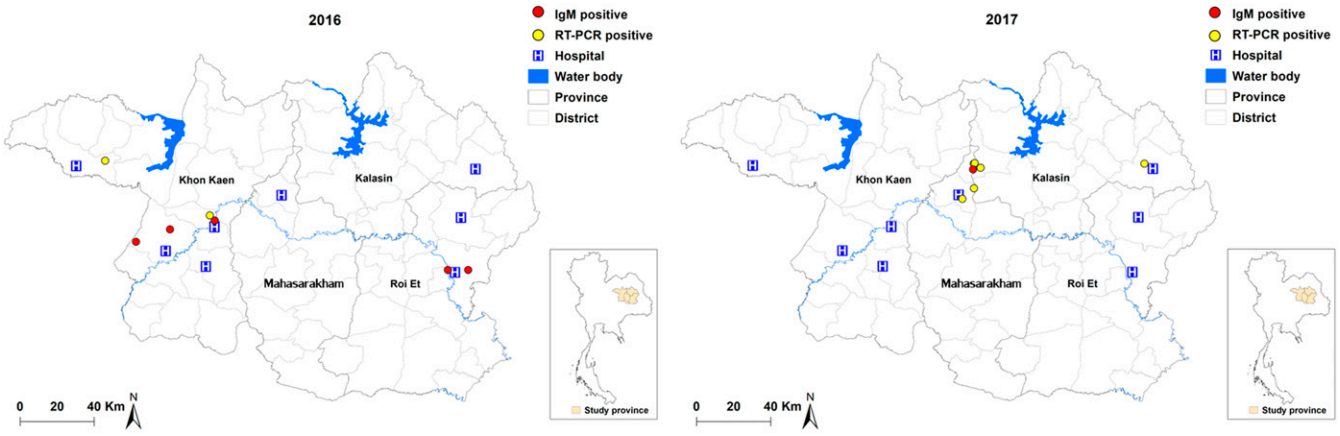

FIGURE 2. Chikungunya virus (CHIKV) infection detected by RT-PCR and anti-CHIKV IgM distribution by study district and province between 2016 and 2017. Red circle represents RT-PCR positive cases; yellow circle represents anti-CHIKV IgM positive cases. This figure appears in color at www.ajtmh.org.

TABLE 3
Co-circulation of CHIKV and DENV infection in acute febrile patients detected by molecular and serological methods (n = 161)

	Molecular analysis		Serological analysis	
	CHIKV-RNA positive	CHIKV-RNA negative	Anti-CHIKV positive	Anti-CHIKV negative
DENV positive (n)–% positive	2 (48)–4.2%	46 (48)–95.8%	9 (60)–15.0%	51 (60)–85.0%
DENV negative (n)–% negative	6 (113)–5.3%	107 (113)–94.7%	14 (101)–13.9%	87 (101)–86.1%

CHIKV = chikungunya virus; DENV = dengue virus.

presented with anti-CHIKV IgG only, which might represent either a previous CHIKV infection with a detectable residual IgG titer or an elevated immune IgG response to a more recent subsequent infection. Because it is assumed that CHIKV antibodies are protective against subsequent infections, in such circumstances, it would be beneficial to investigate the presence of active neutralizing antibodies that might explain the apparent lack of protection to reinfection.

In Asia, CHIKV (along with DENV and other viral agents) is a common, but seldom diagnosed, cause of acute febrile illness in children.[53] The majority of samples (n = 161) with evidence of current or prior CHIKV infection were from < 15-year-old individuals (65.8%) (n = 106). Within this younger age cohort, the distribution between anti-CHIKV IgM, anti-CHIKV IgG, and CHIKV RNA was 5.7%, 3.8%, and 6.6%, respectively. Anti-CHIKV IgM was only present in those aged < 15 years. When viewing exposure history alone, 25.5% of those ≥ 15 years were positive for having CHIKV infection, whereas only 16% of the < 15 years age-group had evidence of infection. The 24–64 years age interval showed the greatest CHIKV infection (current or prior), 44.8% of the sample (n = 29), with 92.3% of those positive having detectable anti-CHIKV IgG. This finding is consistent with other studies showing the long-term persistence of anti-IgG and neutralizing antibodies in adults,[16,17,33,38,52] suggesting higher protective immunity at older age derived from previous CHIKV exposure. Similar observations on infection age distribution and younger age-groups have been reported.[22,54,55] Males and females were similar in percent infection from patients tested, 15% and 22%, respectively. These findings are similar to other observations in Thailand.[26,33] Any difference between gender may have a valid biological basis or simply be biased by sampling method (hospital-based), behavior, occupation, or some other factors separating genders by exposure risk. The information provided in this study alerts medical professionals of the importance of under- and misdiagnosed CHIKV infection in addition to the difficulty of recognizing chikungunya-related clinical symptoms in younger children with acute and persistent arthralgia and myalgia.[56,57]

This study demonstrates the fairly uniform and widespread distribution of CHIKV infection in the northeastern region of Thailand. Six hospitals showed the presence of recent or active CHIKV infection based on the presence of anti-CHIKV IgM and qRT-PCR findings during 2016–2017. These findings are in agreement with other recent serological observations in the country[33,58] and elsewhere in South and Southeast Asia.[13,14] The incidental findings of CHIKV infections in dengue-suspected case surveillance suggest a substantial underlying incidence of infection, thus increasing the risk of reemergence of virus transmission.

There have been only a few published studies on the presence of CHIKV infections in field-collected *Aedes* vector mosquitoes in Thailand.[39–41] The contemporaneous presence of CHIKV in *Ae. aegypti* and humans demonstrated local transmission and once again illustrated the potential epidemic risk with the reemergence and potential expansion of CHIKV in northeastern Thailand. Entomological investigations were performed in and around houses of suspected dengue cases and neighboring houses. Adult *Ae. aegypti* represented 97.8% of the collected *Aedes* species, wherein two mosquito pools (2.9%) had CHIKV RNA, indicating *Ae. aegypti* is the primary vector of CHIKV in the surveilled areas. Similarly, in southern Thailand, 3.3% of field-caught *Ae. aegypti* female mosquitoes were infected with CHIKV during an outbreak period.[40] In general, the number of *Ae. albopictus* collected in mostly urbanized settings was too small to make any determination if this species might play a larger role in transmission in other locations of Thailand. These findings are comparable with a study in southern Lao PDR (Champasak Province), which detected one CHIKV-positive pool (10 female *Ae. aegypti*) by RT-PCR, of which two mosquitoes were positive from 2,003 individuals assayed.[20] In Gabon (western Africa), CHIKV infection in *Ae. albopictus* was relatively low, with a minimal infection rate of 0.3% based on only two positive female pools.[59] These findings are consistent with other observations showing the presence of natural virus-infected mosquitoes is highly variable and often focally distributed but typically very low proportionally to the mosquito population at large.[60–62] Our study was not able to provide complete genetic characterizations of CHIKV from infected mosquitoes. However, other studies in Thailand showed CHIKV in *Ae. albopictus* from west-central Thailand belonged to the ECSA genotype[39] and that virus detected in *Ae. aegypti* from 27 provinces of Thailand collected between January and June 2019 were from the Indian Ocean clade (IOL) and East/South African clade.[39–41]

Concurrent transmission of DENV and CHIKV has been reported in Thailand[33,63] and other countries.[64,65] Therefore, it was not surprising to discover both viruses co-circulating in our study area. From 40 positive dengue patients, qRT-PCR revealed two samples (4.2%) as CHIKV-DENV coinfections. There was no evidence that these dual infections presented more severe illness or pathological outcomes than mono-infections. However, the presence of both viruses emphasizes the need for sustained preventive action to control transmission using the same vector abatement methods of larval source management and adult suppression to reduce infection risk.

This investigation provides a genetic characterization of CHIKV infections during the study period. Because of the low viral load in plasma, only one strain provided sufficient amplification of the *nsP1* gene (447 bp). The strain THAI/KhonKaen/CP4005-8/2016 closely matches (97.5%) the ECSA genotype and is phylogenetically nearest the IOL. This patient was also coinfected with DENV based on qRT-PCR detection. The patient (5-year-old boy) presented on day 2 after symptomatic (fever) onset, which helps explain the initial

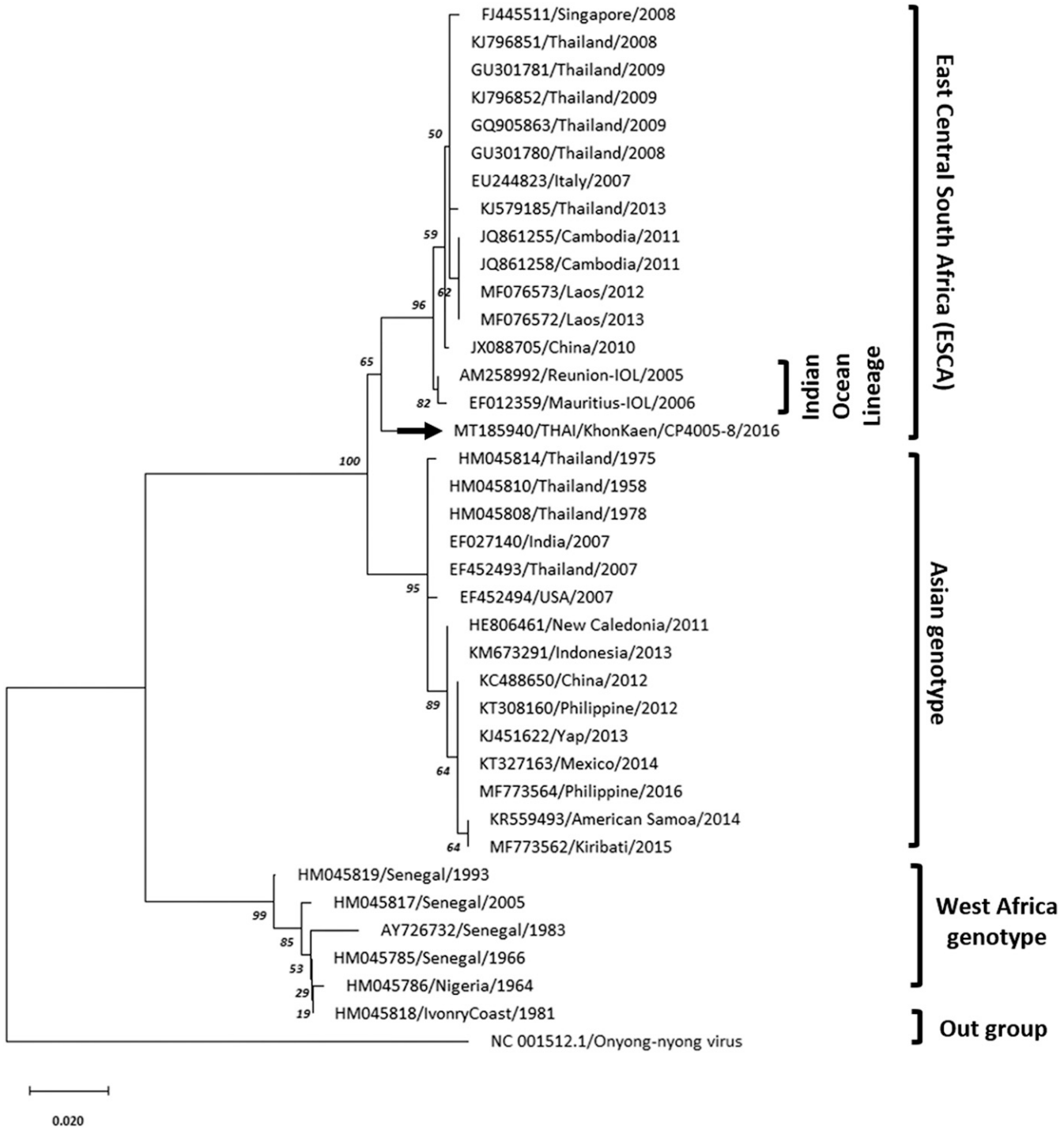

FIGURE 3. Phylogenetic tree for partial chikungunya virus (CHIKV) nonstructural protein 1 gene nucleotide sequences. The phylogenetic analysis used 36 reference strains and one outgroup (O'nyong-nyong virus) and constructed by using the neighbor-joining method. All sequences labeled by Genbank accession number/country/year of isolation. The black arrow represents the CHIKV strain detected in this study.

absence of anti-CHIKV IgM/IgG. These findings are comparable with the detection of persistent neutralizing antibodies against CHIKV-ECSA strains in Chum Phae district.[38] These data confirm the persistent and low-level circulation of CHIKV in the human population of northeastern Thailand following the last recorded outbreak more than 20 years ago. Phylogenetic analysis reveals the Chum Phae strain is most closely related to the strains isolated from previous outbreaks in Lao PDR in 2012–2013,[20] Cambodia in 2011,[18] and China 2010.[66] The CHIKV strain reported in this investigation might be derived from strains found in Thailand during the 2008–2009 ECSA (IOL),[26,28,32,37] 2010,[27] and 2013[32] outbreaks. Although the first CHIKV isolates in Thailand were the Asian genotype,[8] the current and prevailing strain in circulation appears to be the ECSA genotype, beginning with the large outbreak in southern Thailand in 2008.[23]

This study presents some inherent limitations for extrapolation of findings. First, population coverage was spatially restricted to four provinces in northeastern Thailand and only focused on symptomatic cases of suspected dengue infection detected at select sentinel hospitals. Passive collection mechanisms likely contributed only a small percentage of actual infections in the general population. This study design missed asymptomatic and mild (nonfebrile), subacute infections and those seeking medical assistance elsewhere outside the larger public providers. Furthermore, the hospital-based study may have resulted in selection bias with regard to detecting a wider range of circulating CHIKV strains in the region. Second, the low viral load in plasma samples affected fragment amplification and sequencing, thus limiting phylogenetic analysis. All attempts to isolate virus from plasma using a Vero cell line were unsuccessful. In future, virus isolation from serum should be attempted using other susceptible vertebrate and mosquito cell lines for cultivation of CHIKV such as HeLa, BHK-21, C6-36 *Ae. albopictus* and *Toxorhynchites amboinensis*.[8,67,68] For mosquito testing, adult female samples were separated into pools according to cluster, not by individual household; therefore, it was not possible (or the intent of this study) to determine if there were distinguishing differences in CHIKV infection "risk" between patients and surrounding houses. An assessment of *Aedes* vector adult densities and other entomological findings (e.g., adult and immature stage indices) in relation to transmission risk will be presented in a follow-up article. In the study area from where we obtained the mosquito samples, *Ae. albopictus* was seen infrequently in proportion to *Ae. aegypti* (97.8% of adult collections) and collection took place in predominately urbanized settings. However, some areas did have higher numbers of *Ae. albopictus* to be presented in another article. No attempt was made to sequence the virus from the two CHIKV mosquito pools. This would have added further evidence to the phylogenetic assessment of CHIKV strains circulating in the study area. Last, more specific measures of signs and symptoms with patient follow-ups for CHIKV infections (and DENV coinfections) might have been helpful to differentiate between CHIKV and DENV mono-infections, especially evidence of chronic arthritis, a notable residual ailment following CHIKV infection.[2]

In conclusion, this study appears to be the first demonstration of the detection of concurrent CHIKV infection in humans and mosquitoes using hospital-based surveillance in northeastern Thailand. Although the PCR-confirmed CHIKV infections in human and mosquito samples were relatively few, they occurred in all four districts. The one successfully sequenced strain indicated an ECSA genotype–IOL origin, a finding in agreement with other recent studies in Thailand. Given the limited information available on CHIKV occurrence and epidemiology in Thailand, this study contribution clearly indicates that chikungunya must be considered a possible etiology in the clinical differential diagnosis of acute febrile illness. Coinfection with CHIKV and DENV confirmed *Ae. aegypti* as a dual virus transmission threat. Together with the DENVs and the potential reemergence of Zika virus in Asia, increased clinical and laboratory capacity is needed to accurately diagnose and differentiate between these three viral infections. Moreover, as the public health prevention and control methodologies are identical for all three *Aedes*-borne pathogens, those that primarily focus on vector mosquito abatement, an increased capacity for early detection, and response to local transmission are essential for mitigating the threat of outbreaks.

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