INTRODUCTION

Chikungunya (CHIK) fever is a re-emerging mosquito-borne viral disease characterized by abrupt onset of a high-grade fever, severe arthralgia, followed by maculopapular rash; the disease is often self-limiting requiring minimal supportive care and is rarely fatal. Arthralgia associated with CHIK fever is often debilitating and usually resolves within days to a few weeks, but in some cases, may last for months or years; sporadic cases of neurological complications and complications in pregnant women have been reported and especially noted in the Indian Ocean re-emergence of CHIK in 2005.3,4

Chikungunya virus (CHIKV) is a single-stranded, positive-sense RNA, enveloped virus, and a member of the genus Alphavirus of the Togaviridae family.5 The virus is transmitted to humans by numerous Aedes mosquito species, including Aedes (Ae.) aegypti and Aedes albopictus.6 These vectors are ubiquitously found throughout the tropical and subtropical world and have more recently emerged in Europe and the United States.7

Chikungunya shows high epidemic transmission followed by long dormant periods. It was first isolated from both humans and mosquitoes in Tanzania in 1953 and has since caused sporadic outbreaks in Africa and Southeast Asia; in Asia, CHIK was documented first in Bangkok, Thailand in 1958, and was identified as the Asian lineage. This same lineage caused outbreaks in Cambodia, Vietnam, Malaysia, and India during the 1960s.8 In India, CHIKV transmission continued until 1964, reappeared in the 1970s, and declined by 1976. Subsequently, small outbreaks were reported in Africa and Asia between 1976 and 2004 (reviewed in Powers 2007).9

Recently, a large outbreak emerged in the islands of the South West Indian Ocean in the French islands of La Reunion, Mayotte, Mauritius, and Seychelles, which have reported sustained transmission since 2005.8–10 After nearly three decades, CHIKV re-emerged in Southeast Asia with significant outbreaks reported in India, Singapore, Malaysia, and Thailand.11–14

In August 2008, CHIK re-emerged in the southern provinces of Thailand with 49,069 cases reported by the end of 2009. Clinical manifestations and routine laboratory tests are not specific to CHIKV infections and the disease is difficult to differentiate from dengue fever, leptospirosis, scrub typhus, and malaria that are concurrently epidemic in Thailand. Diagnostic tests for CHIK including viral isolation, molecular assays, and serologic tests are well established; however, the viral kinetics and antibody responses are not well characterized. In this study, we attempted to characterize clinical characteristics of CHIKV infections and determine the kinetics of CHIKV viremia and the antibody response using serially collected samples and clinical observations of known laboratory-confirmed CHIKV-infected individuals during the outbreak occurring in Southern Thailand.

MATERIALS AND METHODS

Our study enrolled subjects ≥18 years of age who presented with acute fever and joint pain within 4 days of reporting to Thepha Hospital, a primary care hospital in Songkhla Province, Thailand between April and June 2009. The Ethics Committee of Thepha Hospital approved the protocol and all subjects signed informed consent. Subjects were asked to return to the hospital every 2 or 3 days for the first 10 days and then again 3 weeks after the initial visit. Core body temperature, characteristics of joint pain and rash were evaluated and blood samples taken at each visit. A board certified rheumatologist evaluated joint pain and a severity score, based on the subjects’ limitation of activity, was applied at each evaluation. The severity score was applied as follows: 5, bed ridden; 4, severe limited activity and unable to care for themselves; 3, limited ability to care for themselves; 2, full ability to care for themselves, but unable to work; 1, limited pain but able to return to work; and 0, pain free. Complete blood cell count, renal function tests, and liver function tests were also performed at the first visit. To determine viral and antibody kinetics, polymerase chain reaction (PCR) and serological tests were performed for each available blood sample. All subjects were not fully compliant and did not return to the
hospital as requested; the distribution of samples available for analysis are shown in Figure 1.

**Molecular analysis.** All serum samples were initially tested and screened with a sensitive CHIKV-specific nested PCR. Quantitative PCR (qPCR) was used to determine the magnitude of the viremia. Viral RNA was extracted from 140 μL of patient serum using the Qiagen Viral RNA extraction kit (Valencia, CA) per manufacturer instructions.

Chikungunya virus was identified from patient serum using a two-step nested PCR to amplify the capsid region of CHIKV originally described by Porter and others.\(^{15}\)

**Quantitative reverse transcription-polymerase chain reaction (RT-PCR).** Extracted viral RNA from serum was quantified using the qPCR protocol developed by Smith and others.\(^{16}\) using primer and probe sequences specific to the nsP2 gene. Dr. Dacri Smith and Dr. John Lee USAMRIID, Fort Detrick, Maryland provided positive control and viral standards. The plaque-forming unit (PFU) quantified RNA was diluted 10-fold to the equivalent of 1 PFU to develop the standard curve. All qPCR reactions were performed on the ABI 7300. The Ct values were quantified using ABI software and the standard curve. Duration of viremia was assessed by determining the number of successive days positive assuming that days without blood draws remained positive if detectable viral RNA was present on both sides of the missing day and endpoints were determined as the last day positive if the Ct corresponded to less than or equal to 100 PFU. Any patient without a clear endpoint was excluded from analysis. Statistical analysis of qPCR viral load magnitude by day were calculated using Kruskal-Wallis non-parametric one-way analysis of variance followed by Dunn’s multiple comparisons post test.

Selected CHIKV isolates were sequenced using primers specific for E1 gene. All sequences generated were deposited in GenBank with accession nos. JN661148–JN661156.

**Anti-CHIKV IgM/IgG EIA.** Ninety-six-well microtiter plates (Linbro/Titertek, MP Biomedicals Santa Ana, CA) were coated with 100 μL/well of 1:400 (0.25 μg/well) of Goat anti-mouse IgG (KPL, Gaithersburg, MD) (A) and Goat anti-human IgM (B) or anti-human IgG (C), in 0.018M Carbonate buffer pH 9.0. The plates were incubated at 4°C, overnight. Each plate (A and B or C) was blocked with 1% normal goat sera (NGS) in 0.5% casein-0.5% bovine serum albumin (BSA) in phosphate buffered saline pH 7.4 (PBS) [Buffer B] for 2 hr at room temperature. Fifty μL of K42 monoclonal antibody anti-CHIK diluted at 1:1,000 in Buffer B then added and incubated for 2 hr at 37°C. The plates were washed with phosphate buffered saline (PBS) with 0.05% Tween 20 and 50 μL of 50 HA U/well of CHIK antigen diluted in Buffer B was added. After incubation at 4°C overnight, the plates were washed and 50 μL/well of either patient serum (1:100, 1:1,000) in A or Purified human IgM or IgG in B or C (for Std curve) diluted in Buffer B. Each plate was incubated for 2 hr at room temperature. The plates were washed and 30 μL/well of optimum dilution (determined for each assay) of horse-radish peroxidase conjugated Goat anti-human IgM or IgG diluted in Buffer B was added and incubated at 37°C for 1 hr. After washing, 100 μL/well of SureBlue TMB (KPL) was added. After 10 minutes a 50 μL/well of 0.2 H₂SO₄ was added to stop color development and plates were read at A450/650 (Softmax Pro). Negative and positive standard controls were included in every plate. Results are expressed as μg/mL (calculated from standard curve and dilution factor).

**Hemagglutination-inhibition serology test.** Hemagglutination inhibition serology for antibody to CHIKV was performed on acetone extracted serum samples by using the procedure of Clarke and Casals and was modified for 96-well V-bottom plates.\(^{17}\) All sera from the same individual were tested on the same plate. Sixteen units of sucrose acetone extracted suckling mouse brain CHIKV antigen was add into 25 μL of 2-fold serial dilution of serum (1:10 to 1:1280 dilutions). After overnight incubation at 4°C, 50 μL of goose red blood cell in phosphate buffer was added and incubated at 37°C for 1 hr. A patient was diagnosed as having acute CHIK infection if there was a 4-fold or greater increase in CHIK antibody titer between paired serum specimens.\(^{18}\)

**Plaque reduction neutralization assays (PRNT50).** The PRNT assays were conducted on available samples using methods described by Russell and others.\(^{19}\) In brief, an equal volume of diluted antisera, 4-fold serial dilution of antisera (1:10, 1:40, 1:160, 1:640, 1:2560) were incubated with an equal volume of three CHIKV strains at a concentration of 100 PFU/mL, at 35°C for 1 hr. Confluent LLC-MK₂ cell monolayer in 12-well plates (Corning, Tewksbury, MA) were washed once with HBSS (Life Technologies, Carlsbad, CA) and incubated with a 100 μL of virus-sera mixtures on a rocker platform for 1 hr at room temperature. The virus-sera mixture inocula were removed and 1 mL of a first overlay media consisting of 1.8% of low-melting point agarose (LMP), (GIBCO) equally mixed with 2X modified Eagle’s medium (GIBCO) adjusted pH to 8.2 was added to each well. The plates were incubated at 35°C in an incubator with 5% CO₂ for 4–5 days. A 1 mL of second overlay media, 1.8% LMP equally mixed with 2X modified Eagle’s media, without HIFBS/NaHCO₃ including 0.01% neutral red (SIGMA, St. Louis, MO) was added to stain plaque and ease for counting. The plates were incubated at 35°C and viral plaques were counted over a light box the following day. Each sample was run in duplicate and all assays were conducted in a biosafety level 3 laboratory. Three CHIK Strains were used 1) CHIK SV0451/96, a Southeast Asian strain isolated in

![Figure 1](image-url)
Thailand during the 1996 epidemic; 2) CHIK SV2910/09 an East African/Indian Ocean Strain isolated from Thailand during the 2008/2009 epidemic; and 3) the CHIK vaccine strain 181/clone25 (181/25) developed by the United States Army Medical Research Institute of Infectious Diseases (USAMRIID).²⁰ The PRNT₅₀ titers were determined by probit analysis and results with multiple samples are presented as geometric mean titers.

RESULTS

We examined 45 subjects with acute fever and joint pain. All were confirmed CHIK positive by PCR and by seroconversion using hemagglutination inhibition assay (HAI), IgM/IgG enzyme-linked immunosorbent assay (ELISA), and/or PRNT. The mean age of the subjects was 49 years with a male/female ratio of 1:1.4. Thirty-five subjects (77.8%) were gardeners, mostly working in the abundant rubber tree plantations within Songkhla Province. Eight subjects, (17.8%) were found to have underlying disease and the most common being hypertension (Table 1). All subjects had fever and joint pain, as required by inclusion criteria and reported to the hospital a mean 1.2 days after the onset of illness with a mean fever duration of 5.5 days. As shown in Table 2, all subjects’ experienced joint pain during their disease and 42 (93%) involving multiple joints. Interphalangeal joints were the most common joint affected and observed in 41 (91%) subjects. Also commonly affected were the joints of knee (71%), elbow (67%), and wrist (56%). The mean duration of severe joint pain (score 4 or 5) was 5.8 days but 11 (25%) subjects reported joint discomfort (score of 1 or 2) through the convalescence visit, up to 29 days post onset of symptoms. Rash was observed a mean 3.5 days post symptoms onset in 37 (82%) subjects. The trunk (53%) and limbs (52%) were the most common sites but several subjects had an observed rash on the face (Table 2).

Routine laboratory tests conducted at the initial visit included a complete blood count and renal and liver function tests. Mean values are shown in Table 3. Twenty-one subjects (46%) exhibited lymphocytopenia and 5 (11%) subjects were leukopenic. six subjects were thrombocytopenic with platelet counts of < 150,000 cell/mm³. Liver enzymes were elevated in 15 (33%) subjects. The remaining results of the routine laboratory tests fell within normal ranges or were unremarkable for all subjects (Table 3).

The CHIKV E1 gene sequencing of viruses extracted from patient serum samples confirmed the East African strain containing the A226V mutation to be the infecting agent and similar to the strain circulating throughout countries in the Indian Ocean (Figure 2). Phylogenetic analysis of selected samples collected in this study and the full lengths E1 sequences available in GenBank revealed strains from our study fell within a clade of Malaysian sequences collected between 2008 and 2009 and samples unrelated to this study collected in Thailand.

Both sensitive nested PCR and qPCR assays were used to detect and quantify CHIKV RNA. The nested PCR used to determine the overall positivity and qPCR was used to determine the magnitude of the viral load in each serum sample. As shown in Figure 3A, all samples tested positive for CHIK by nested PCR collected during the first 4 days of infection. The first round PCR positivity decreased proportionately each day from fever onset but remained positive in the second nested PCR in samples collected through Day 9. Subjects were positive by nested PCR for a mean of 5.9 days (95% confidence interval [CI] 4.9 to 6.9 days) range 3–14 days. Viral load reached its maximum level before or shortly after enrollment. The mean viral load was maximal on Days 1–5 and was statistically different with 5 to 6 logs 10 PFU/mL of viral RNA from Day 6 dropping to 2.6 log 10 PFU/mL. One subject remained positive through Day 9 and one subject as long as 14 days albeit with a low but detectable 10 PFU/mL in this patient (Figure 3B).

The rapid decrease in viral load corresponded to the rise of anti-CHIK IgM antibodies at Day 6. As shown in Figure 4, IgM antibodies appeared on Day 4 after fever onset peaking at Day 7, and dropped off rapidly but sustained levels were detected through Day 14. Anti-CHIK IgG antibodies first appeared at Day 5 and rose steadily through Day 24. The IgG curve was reproduced using a Hemagglutination inhibition assay (Figure 4). Neutralization assay using three chikungunya strains confirmed the kinetic antibody curve of the ELISA and HAI data. No subjects exhibited neutralizing titers in the first 10 days of infection for any of the three strains. A slightly
Table 3
Routine laboratory data of subjects with acute chikungunya infection*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Normal range</th>
<th>Subjects (N = 45)</th>
<th>Subjects with abnormal laboratory results</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC count, cells/mm³</td>
<td>4000–10,000</td>
<td>5987 ± 1734</td>
<td>&lt; 4000:5</td>
</tr>
<tr>
<td>PMN count, cells/mm³</td>
<td>1500–7500</td>
<td>4098 ± 1945</td>
<td>&lt; 1500:0</td>
</tr>
<tr>
<td>Lymphocyte count, cells/mm³</td>
<td>1000–4000</td>
<td>725 ± 455</td>
<td>&lt; 1000:21</td>
</tr>
<tr>
<td>Platelet count × 10³, cells/mm³</td>
<td>150–450</td>
<td>196 ± 72</td>
<td>&lt; 150:6</td>
</tr>
<tr>
<td>Creatinine level, mg/dL</td>
<td>0.8–1.2</td>
<td>1.1 ± 0.8</td>
<td>&gt; 1.2:6</td>
</tr>
<tr>
<td>AST level, U/L</td>
<td>10–40</td>
<td>29 ± 18</td>
<td>&gt; 40:15</td>
</tr>
<tr>
<td>ALT level, U/L</td>
<td>10–40</td>
<td>35 ± 11</td>
<td>&gt; 40:10</td>
</tr>
<tr>
<td>ALP level, U/L</td>
<td>10–100</td>
<td>75 ± 24</td>
<td>&gt; 100:0</td>
</tr>
</tbody>
</table>

*WBC = white blood cell; PMN = polymorphonuclear neutrophil; AST = asparate aminotransferase; ALT = alanine aminotransferase; ALP = alkaline phosphatase.

Figure 2. Maximum likelihood phylogenetic trees of selected chikungunya samples isolated from subjects in Songkhla Province Thailand. All strains were identified as the East African/Indian Ocean stains and contain the A226V mutation. Scale bar indicates the number of substitutions per site. Bootstrap support values are shown for key nodes only.
higher response was observed with the CHIK vaccine strain 181/clone25 (181/25) than the homologous East African/Indian Ocean strain isolated from the current Thailand outbreak or the Southeast Asian strain isolated in 1996, as shown in Figure 5. There were no associations found between the level of neutralizing antibodies with duration or severity of arthralgia.

DISCUSSION

The first record of CHIK outside Africa was in Bangkok during the epidemic of dengue hemorrhagic fever in 1958.21 Since then, there have been eight sporadic outbreaks of this disease in Thailand22–25, this outbreak is the largest recorded in Thailand. Chikungunya fever presents with similar symptoms to other disease endemic in Thailand including dengue fever, leptospirosis, scrub typhus, and malaria. Proper clinical management of this disease requires pathogen identification using specific molecular and serological diagnostic tests to differentiate CHIK from other etiologies. A better understanding of Chikungunya virus, the clinical disease manifestations, and the immune response to virus is needed for improved clinical management, diagnostic and therapeutic development, and better disease identification in the absence of laboratory tests.

This study recruited subjects into the study based on a strong suspicion that they were infected with CHIKV. Inclusion criteria required that all subjects have fever and arthralgia. All subjects reported to the hospital within 3 days with fever and arthralgia. Of the 45 subjects, 93% showed signs of polyarthralgia, with varying degrees of severity and involving the small joints including the finger, knee, elbow, and wrist. These symptoms are similar to recent studies of CHIK outbreaks in India, Singapore, and Reunion Island.26–30 In our study, only one-third of the subjects presented with symmetrical arthralgia, which was slightly lower than recent CHIK studies31; we applied a pain score in evaluation ranging from five, effectively bed ridden to one, able to work but with some limited pain. The majority, 33 of 45 (78%), of subjects entered the study with polyarthralgia with a pain score of five and achieved a pain score of one by Day 14 and resumed normal activity. Pain associated with joints had completely diminished for most subjects by the last visit however, 10 subjects reported mild pain 22–31 days after the onset of symptoms. Of interest, the larger joints of the knee, elbow, wrist, and ankle appeared to be the longest afflicted. There are reports

Figure 3. (Panel A). The percent positive samples using the two-step nested polymerase chain reaction (PCR) technique. The reverse transcription-PCR (RT-PCR) is the first step and is a qualitative assessment of viral load (black bars). Samples positive by RT-PCR represent a higher viral load then samples only positive in the second nested PCR step (grey bars). (Panel B) Quantitative PCR using extracted RNA fromittered viruses. Results are expressed as plaque-forming unit (PFU)/mL. Error bars represent the upper standard error of the mean.

Figure 4. Serological responses to chikungunya infections. Anti-chikungunya immunoglobulin M and G (IgM and IgG) were measured using enzyme-linked immunosorbent assay (ELISA) and healthcare-associated infections (HAI). The quantitative PCR result is included in the graph to show antibody response in relationship to viremia. The error bars for the serological results reflect the standard error of the mean.
of similar finds with severe arthralgia diminishing within the first 2 weeks. However, significant numbers of CHIKV-infected persons experienced relapsing and continuous moderate joint pain and discomfort months and years after infection.2,32 Interestingly, several subjects in our study reported joint pain before the onset of fever, however this did not appear to have any relationship with severity or persistence of arthralgia.

All 45 subjects reported fever with a mean temperature of 39.5°C at enrollment and through the first 3 days and returning to normal for most subjects by Day 8. The mean fever duration of 5.9 days was consistent with many studies, although fever duration of nearly 2 weeks was reported.9,27,30,33 Skin rash appears to be variable in CHIK infections ranging from 14% in 1965 Madras City study to 75% in Reunion Island and rash appears to enhance transmissibility of the virus by shortening the intrinsic incubation period in the vector.38,39

The CHIKV strain identified in this study was the East Central South African (ECSA) strain. The ECSA contains the A226V mutation in the E1 gene consistent with the strain identified in other studies conducted in Thailand and throughout the Indian Ocean region since 2005.3,13,36,37 The mutation appears to enhance transmissibility of the virus by Aedes albopictus by increasing replication and dissemination rates and thus shortening the intrinsic incubation period in the vector.58,59 In this study, the majority of the subjects were farmers primarily working on rubber tree plantations, which are numerous in Southern Thailand. These plantations are a prime breeding ground for Aedes albopictus, which are better adapted to peridomestic settings with vegetation that provides its preferred larval development and resting sites than Aedes aegypti.40 Aedes albopictus being the dominate vector may contribute to the observation that the Thai outbreak is disproportionately affecting more adults when compared with children and older working class individuals in the rubber plantation fields, although children would likely be more exposed to A. aegypti in homes and schools.14 Other reports have also found more adults symptomatic with CHIK and with increased severity compared with children.29,29,41

Significant gaps in the literature exist for viral and serological kinetic data in relation to human CHIKV infections. In this study, we attempted to fill some of these gaps with a relatively large sample size but were limited because our study was an outpatient protocol and serially collecting specimens from subjects on their convenience based on the physicians’ guidance of returning to the hospital every 2 or 3 days. Nonetheless, our data were consistent with experimentally controlled CHIKV experiments conducted in cynomolgus macaques, as described in a paper by Labadie and others;42 in our study, the peak viral titer occurred in the first 5 days with a mean RNA copy equating to 6 log PFU/mL. Samples collected as far out as Day 12 were positive but viral RNA found a mean length of 6 days using nested PCR and 5.4 days using the slightly less sensitive qPCR. Labadie showed that monkeys infected with 10^3 PFU of virus viremia peaked at Day 2 and persisted until Days 6 or 7. The viremic period in our study was longer than that determined in a Reunion Island study, which showed a decrease in viremia in all cases between Days 1 and 3 and all subjects negative by Day 6. However, it was similar to a study conducted in Singapore with at least 6 days of viremia.31,43 It is impossible to determine the infectious dose that subjects received in this study; however, it appears that humans naturally infected maintain a higher viremia for a longer period than monkeys.

Anti-CHIK IgM was detectable in all subjects, appearing as early as Day 3 since the onset of symptoms and persisting at high levels through the last blood draw at Days 22–33. The IgM appearance during the early acute phase is well documented and often used as a diagnostic test for CHIKV infections.30,44 Anti-CHIK IgG levels appeared to coincide
with the appearance of IgM in some cases but positive in all patient samples after Day 6, because the onset of symptoms occur at the point when the viral titers are rapidly declining. The appearance of IgG was much earlier than expected and might possibly indicate memory from previous CHIK infections or infections for a different alphavirus; however, similar findings of anti-CHIK IgG detected early in the disease have been documented in recent studies. In light of these results the kinetics of antibody responses to CHIKV infections need additional study.

We believe that this is one of the first studies to attempt to characterize the kinetics CHIK infections in humans. Understanding the kinetics of clinical and laboratory aspects of CHIK infections have important implications in treatment and diagnosis of this debilitating disease. These studies are difficult to conduct in an outpatient setting and need to be considered during hospitalization. The need for frequent blood draws and careful patient observations and clinical evaluations are critical to gain a full understanding of CHIK in humans.

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REFERENCES


