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Heterogeneity of clinical isolates of chikungunya virus and its impact on the responses of primary human fibroblast-like synoviocytes

Apamas Sukkaew,[1] Montri Thanagith,[2] Tipparat Thongsakulprasert,[2] Margit Mutso,[3] Suresh Mahalingam,[3] Duncan R. Smith[4,5] and Sukathida Ubol[1,4,*]

Abstract

Low-passage clinical isolates of chikungunya virus (CHIKV) were found to be a mixture of large- and small-plaque viruses, with small-plaque viruses being the predominant species. To investigate the contribution of plaque variants to the pathology of the joint, primary human fibroblast-like synoviocytes (HFLS) were used. Large- and small-plaque viruses were purified from two clinical isolates, CHIKV-031C and CHIKV-033C, and were designated CHIKV-031L and CHIKV-031S and CHIKV-033L and CHIKV-033S, respectively. The replication efficiencies of these viruses in HFLSs were compared and it was found that CHIKV-031S and CHIKV-033S replicated with the highest efficiency, while the parental clinical isolates had the lowest efficiency. Interestingly, the cytopathic effects (CPE) induced by these viruses correlated with neither the efficiency of replication nor the plaque size. The small-plaque viruses and the clinical isolates induced cell death rapidly, while large-plaque viruses induced slow CPE in which only 50 % of the cells in infected cultures were rounded up and detached on day 5 of infection. The production of proinflammatory cytokines and chemokines from infected HFLSs was evaluated. The results showed that the large-plaque viruses and the clinical isolates, but not small-plaque variants, were potent inducers of IL-6, IL-8 and MCP-1, and were able to migrate monocytes/macrophages efficiently. Sequencing data revealed a number of differences in amino acid sequences between the small- and large-plaque viruses. The results suggest that it is common for clinical isolates of CHIKV to be heterogeneous, while the variants may have distinct roles in the pathology of the joint.

INTRODUCTION

Chikungunya virus (CHIKV) is an arthropod-borne virus or arbovirus that is transmitted to humans by *Aedes aegypti* and *Aedes albopictus* mosquitoes [1]. CHIKV is an RNA virus that belongs to the genus *Alphavirus* of the family *Togaviridae* and causes a viral epidemic disease in humans called chikungunya fever (CHIKF) [2, 3]. The viral genome is composed of a positive-sense single-stranded RNA that is approximately 11.8 kb in size and organized into two open reading frames (ORFs). The 5′-terminus carries a methylated nucleotide cap and the 3′-terminus has a polyadenylated tail. The first ORF encodes four non-structural polyproteins (nsP1-nsP4) that participate in genome replication, RNA capping, polyprotein cleavage and other functions required for viral replication. The second ORF encodes five structural proteins, namely the capsid protein (C), two major envelope glycoproteins (E2 and E1) and two small proteins (E3 and 6K) [4]. CHIKV is classified into three distinct phylogroups, the Asian, the West African and the East, Central and South African (ECSA) genotypes [5]. The disease was first recognized in 1952 in Tanzania [6] and the virus caused outbreaks in many countries, including Thailand, from the 1960s to the 1990s [7]. Recently, CHIKV has re-emerged widely, with the Asian lineage causing more than 440 000 infections in the Caribbean, South America (Brazil, Venezuela, Costa Rica and Colombia) and North America [8–10]. Currently, there is no licensed vaccine to protect against, or specific therapeutic agent to treat, CHIKV infection.

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Author affiliations: [1]Department of Microbiology, Faculty of Science, Mahidol University, Rama 6 Rd., Ratchatewi, Bangkok 10400, Thailand; [2]Community Medical Unit, Pang Nga Hospital, Pang Nga, Thailand; [3]Institute for Glycomics, Griffith University, Southport, Gold Coast, QLD, Australia; [4]Center for Emerging and Neglected Infectious Diseases, Mahidol University, Salaya Campus, Nakornpathom, Thailand; [5]Institute of Molecular Biosciences, Mahidol University, Salaya Campus, Nakornpathom, Thailand.

***Correspondence:** Sukathida Ubol, sukathida.ubo@mahidol.ac.th

Keywords: Chikungunya virus; plaque variants; arbovirus; large-plaque CHIKV; small-plaque CHIKV.

Abbreviations: cDNA, complementary DNA; IL, interleukin; MCP, monocyte chemoattractant protein; TNF-α, tumour necrosis factor alpha; UTR, untranslated region; W/V, weight/volume.

One supplementary table is available with the online version of this article.

CHIKV infection causes CHIKF, in which infected individuals develop a high fever, headache, skin rash, myalgia/myositis and arthralgia/arthritis [11]. Several cytokines and chemokines are associated with CHIKF disease progression, including type I interferons (IFNs) and pro-inflammatory cytokines (IL-6, IL-8, IL-12, IL-15 and MCP-1), which are increased in patients' sera during acute CHIKF [12], suggesting that a strong inflammatory response of the tissue is one of the immunopathogenic characteristics of CHIKF [13]. In the majority of cases, the signs and symptoms of acute CHIKV usually resolve within 2 weeks [14]. However, a large proportion of patients exhibit a chronic phase, with persistent infection and relapsing severe arthritis that can last several months or even years [15–17], causing considerable disability in the extremities [18]. The viral RNA and proteins can be found in synovial macrophages from these patients for up to 18 months post-infection [19]. Moreover, CHIKV-induced arthralgia/arthritis is associated with musculoskeletal tissue infection [20] and infiltration of immune cells (monocytes, macrophages, NK cells, and $CD4^+$ and $CD8^+$ T cells) to the joints, mediating inflammation [21]. Recently, increased levels of granzyme A, which affected and promoted arthritic inflammation in mice and CHIKF patients, was reported [22].

During the CHIKV outbreak in the southern part of Thailand in 2009–2010, our laboratory successfully isolated CHIKVs from patients' sera. The isolated CHIKVs are composed of a mixed population of small- and large-plaque phenotype viruses with respect to plaque morphology in Vero cells. The large- and small-plaque viruses were plaque-purified to obtain homogeneous large- and small-plaque viruses. How these large- and small-plaque phenotype viruses are involved in CHIKV-induced arthralgia/arthritis is of interest. *In vitro* studies have shown that CHIKV can infect many cell types, such as human epithelial and endothelial cells, monocyte-derived macrophages, primary dermal fibroblasts and synovial fibroblasts [23]. Synovial tissue is a predominant target of CHIKV infection, with severe joint pain or arthritis resulting [19]. Therefore, in the present study we investigated the effects of large- and small-plaque CHIKV in parallel with the clinically isolated parental CHIKV on synovial tissue using primary human fibroblast-like synoviocytes (HFLS) as a model system. We also analysed the genomic sequences of these virus isolates.

RESULTS

Clinical isolates of CHIKV are composed of two different phenotypes of the virus based on plaque size

During an epidemic of CHIKV in the southern part of Thailand in 2009–2010, 6 isolates of CHIKV from patients with severe arthralgia and 12 isolates from patients with mild CHIKF were obtained [24]. These viruses were amplified once in C6/36 cells and the infectious virions were quantified in Vero cells. We observed heterogeneous phenotypes from these CHIKV isolates, with each clinical isolate being composed of large- and small-plaque variants. The large-plaque variants are larger than 4 mm and the small-plaque variants are smaller than 2 mm (Fig. 1a). The number of large- and small-plaques present in each group of isolates was quantified by plaque formation in Vero cells. Strikingly, a high proportion of small-plaque size variants, representing about 70 % of all plaques, was observed from both patient groups, as shown in Fig. 1(b). This may reflect the population of CHIKV in the circulation of patients, and suggests that the small-plaque CHIKV predominated during the outbreak in the southern part of Thailand, warranting further study regarding the reasons behind this characteristic and the roles of plaque variants in the context of the disease pathogenesis.

Small-plaque variants of CHIKVs have higher infectivity in human fibroblast-like synoviocytes as compared to large-plaque viruses and the clinical isolates

To obtain large- and small-plaque viruses, two clinical isolates, CHIKV-031C and CHIKV-033C, were selected. The large-plaque and small-plaque viruses in the clinical isolates were subjected to repeated plaque purification to acquire homogeneous large-plaque and small-plaque viruses. The homogeneous populations of large- and small-plaque variants are shown in Fig. 2(a), alongside their respective parental viruses. These variants were designated CHIKV-031L and CHIKV-031S for the large- and small-plaque viruses purified from the parental CHIKV-031C, and CHIKV-033L and CHIKV-033S for the large- and small-plaque viruses purified from the parental CHIKV-033C. These variants exhibited stable plaque sizes after six rounds of repeat plaque purification.

We hypothesized that different plaque variants naturally occurring in patients have distinct infectivity profiles. As we have previously demonstrated, HFLS cultures are susceptible to CHIKV and produce cytokines that mediate arthralgia/arthritis [25], and so this system was selected as a model system to determine the viral infectivity of the large- and small-plaque variants in parallel with the clinical isolates. Monolayer cultures of HFLS were therefore infected with these viruses at a multiplicity of infection (m.o.i.) of 0.01 p.f.u. $cell^{-1}$, and the viral growth kinetics were monitored for 5 consecutive days. As shown in Fig. 2(b, c), all of the viruses replicated rapidly and yielded progenies up to 10^6 and 10^7 p.f.u. ml^{-1} approximately 48 to 72 h post-infection. The cells infected with CHIKV-031S and, to a lesser extent, those infected with CHIKV-031L produced a significantly higher number of viral progeny as compared to CHIKV-031C. Cells infected with CHIKV-033S produced a significantly higher number of progeny viruses compared to CHIKV-033L and its corresponding clinical isolate. This suggests that the small-plaque phenotype has a growth advantage over the large-plaque and parental viruses in HFLS cells.

The cellular consequences of the infection of HFLS by the CHIKV viruses were also observed. The large- and small-plaque variants from the two CHIKV clinical isolates caused

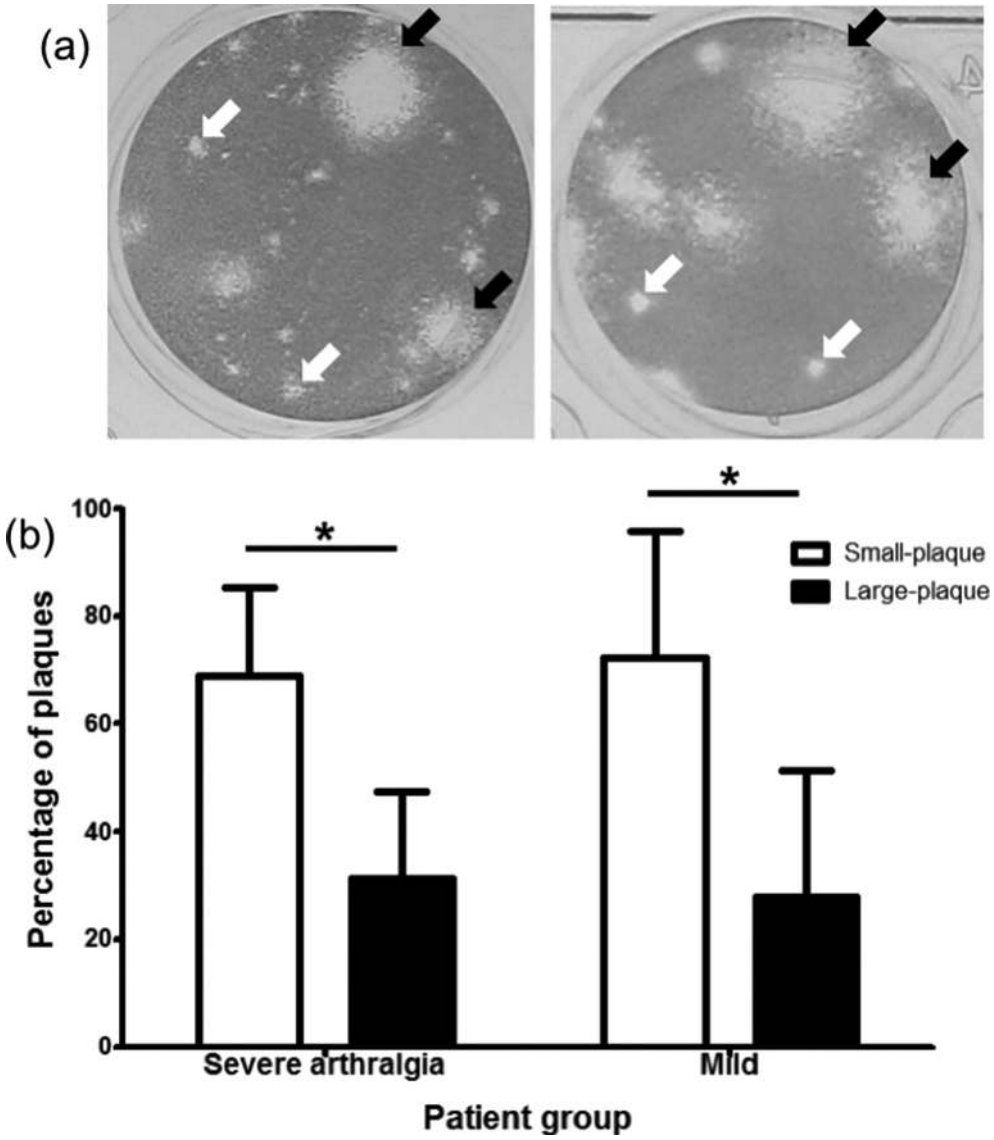

Fig. 1. The percentage of small-plaque variants versus large-plaque variants from clinical isolates of CHIKV. Low passage CHIKV isolates were examined by plaque formation in Vero cells [CHIKV-031C (left) and CHIKV-033C (right). (a) The large-plaque population (size $\geq$4 mm) is indicated with black arrows and the small-plaque population (size $\leq$2 mm) is indicated with white arrows. (b) The CHIKV isolated from the sera of patients with severe arthralgia and mild CHIKF, respectively, were propagated in C6/36 cells, and the supernatants were harvested on day 3 of infection. The number of small- and large-plaque variants in the supernatants was quantified using Vero cells. The data are expressed as percentages of each variant in terms of the total number of plaques. The data showed a mean±SD from n=6 and 12 for severe arthralgia and mild patients, respectively. The data were assessed statistically using Student's t-test with P value <0.05 (two tail).

CPE in HFLS that were similar to those caused by their parental viruses in the sense that the cells rounded up and detached from the culture vessel surface. Interestingly, however, the large-plaque variants produced reduced *in vitro* CPE as compared to the small-plaque variants and the clinically isolated viruses (Table 1). Moreover, the two small-plaque variants exhibited CPE that were indistinguishable from those of the parental viruses. These results indicate that the small-plaque variants have higher infectivity and cause more rapid CPE as compared to the large-plaque variants, suggesting that this could play a major role in CHIKV-induced cellular pathology in infected patients.

Large-plaque, but not small-plaque, viruses are potent stimulators of inflammatory cytokines

As inflammatory mediators contribute significantly to the development of arthralgia/arthritis in CHIKV-infected individuals, the induction of inflammatory cytokines by the different plaque phenotypes of CHIKV was investigated. HFLS cells were infected with small-plaque, large-plaque and

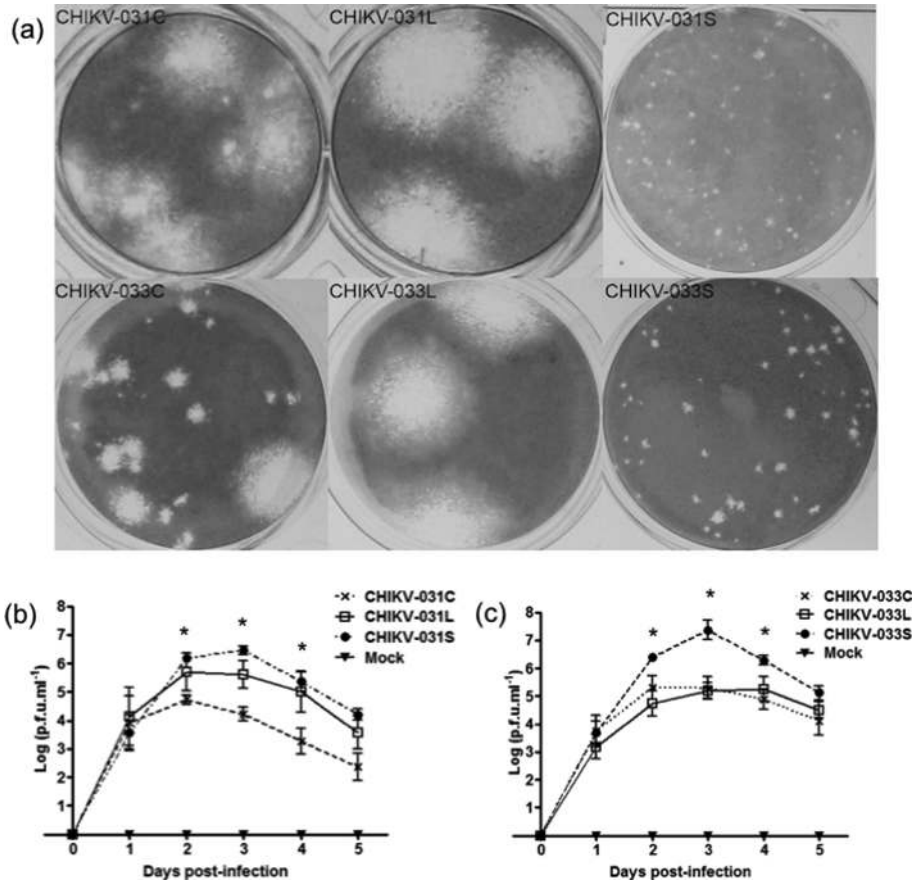

Fig. 2. Plaque morphology and replication kinetics of clinical isolates, large-plaque CHIKV and small-plaque CHIKV. (a) Plaque morphology of clinical isolates, large-plaque variants and small-plaque variants. Human synoviocyte fibroblasts were infected with two isolates of CHIKVs at a multiplicity of infection (m.o.i.) of 0.01. The supernatants of the mock and CHIKV infections were harvested every 24 h after infection for 5 consecutive days. The supernatants were subjected to virus quantification by plaque assay. The growth kinetics of CHIKV-031 and CHIKV-033 are shown in (b) and (c), respectively. The data show the mean±SD from three independent experiments. The data were assessed statistically using Student's *t*-test with *P* value <0.05 (two tail).

clinically isolated viruses at an m.o.i. of 0.01 p.f.u. cell^{-1}. The culture supernatants were harvested for 3 consecutive days after inoculation and quantified for IL-6, IL-8, MCP-1, MCP-3 and TNF-α production using ELISA.

The clinically isolated CHIKVs and large-plaque variants induced IL-6 and MCP-1 in HFLS cells to a significant degree in comparison to mock infection (Fig. 3a–d), while the small-plaque variants did not upregulate these two mediators. None of the tested CHIKVs induced MCP-3 production (Fig. 3e, f). Infection by large-plaque variants or by their parental clinical isolates stimulated IL-8 production significantly, particularly on day 3 post-infection (Fig. 3g, h), while again, in contrast, the small-plaque variants did not induce IL-8 production.

CHIKV-031S upregulated TNF-α production from day 1 of infection to the end of the experiment, while CHIKV-031C only transiently stimulated TNF-α production on day 1 of infection. In contrast, CHIKV-031L did not stimulate TNF-α production (Fig. 3i). CHIKV-033S stimulated the peak of TNF-α production earlier than CHIKV-031S, while CHIKV-033C and CHIKV-033S transiently stimulated TNF-α production (Fig. 3j). These results showed that the clinical isolates CHIKV-031C and CHIKV-033C are weak inducers of TNF-α, while the large-plaque viruses have an undetectable effect on TNF-α production as compared to mock-infected cultures, and the small-plaque viruses are TNF-α inducers.

Large-plaque variants and clinical isolates strongly induce the migration of monocytes/macrophages

Recruitment of inflammatory cells (monocytes/macrophages) to the infected joint is one of the processes leading to arthralgia/arthritis. We performed monocyte migration assays to determine the effects of immunological mediators produced from HFLS cells infected with CHIKV variants. The results demonstrated that supernatants obtained from HFLS cultures infected with CHIKV-031C or CHIKV-031L induced greater CD14^{+} monocyte migration as compared to supernatants from cultures infected with CHIKV-031S

Table 1. Small-plaque variants and clinical isolates of CHIKV produced similar levels of cytopathic effects in HFLS cells

HFLS infected with different variants of CHIKV were observed and graded into five levels determined by the percentage of cell morphology changes: –, no CPE; +, 1–25 %; ++, 25–50 %; +++, 50–75 %; ++++, 75–100 %. The data were obtained from three independent experiments.

Type of infection	Degree of CPE				
	Day 1	Day 2	Day 3	Day 4	Day 5
Mock	–	–	–	–	–
Clinical-isolate 031	–	++	+++	++++	++++
Large-plaque 031	–	–	+	++	++
Small-plaque 031	–	++	+++	++++	++++
Clinical-isolate 033	–	+	++	+++	++++
Large-plaque 033	–	–	+	++	++
Small-plaque 033	–	+	++	+++	++++

(Fig. 4a). For cultures infected with CHIKV-033 variants, infection by CHIKV-033L recruited monocytes/macrophages more efficiently than CHIKV-033C and CHIKV-033S on days 1 and 2 of infection. However, on day 3 of infection, supernatants from the CHIKV-033L and CHIKV-033C cultures exerted a similar level of recruitment, which was significantly higher than that for CHIKV-033S-infected cultures (Fig. 4b).

Sequencing of small- and large-plaque purified viruses revealed differences in amino acid sequences

The sequencing of CHIKV-033L and CHIKV-033S revealed a total of 32 nucleotide changes, with 6 nucleotide deletions being found in CHIKV-033L. Most of the nucleotide differences were located in the coding region and only five were found in the 3′ UTR. Seventeen nucleotide changes were synonymous (Table 2), while nine nucleotide changes resulted in amino acid (aa) changes (Table 3). All of the non-synonymous changes were located in nsP1, nsP2, nsP3 or E2 proteins.

Three aa differences in nsP1 (29 P/S, 146 N/D and 186 N/D) were located in the methyltransferase region, while the 407 L/P aa is located in domain 3 of nsP1. The 1210 T/M aa is located in the RecA-like helicase domain of nsP2. This region was shown to mediate viral RNA synthesis in Sindbis virus, but it does not interact with the nsP2 protease domain [26]. The 1412 P/S is located in the nsP3 macrodomain and is conserved in alphaviruses. 1636I/V and 1687P/L are located at the junction of the zinc-binding and hypervariable regions of nsP3. Interestingly, for CHIKV-033L there were two aa deletions at the end of nsP3, while in the CHIKV-033S virus this position had leucine followed by an opal termination codon. The last aa difference was located in the structural polyprotein 709 G/S position, which corresponds to a region in the E2 C-terminal transmembrane domain.

DISCUSSION

CHIKV is a re-emerging alphavirus that has high potential to emerge in immunologically naïve populations. CHIKV circulates between two host species, the mosquito vectors and the vertebrate hosts, and thus CHIKV is likely to exist as a heterogeneous population. In the present study, we showed that the investigated low-passage clinical isolates of CHIKV exist as mixed populations of small- and large-plaque phenotypes, with small-plaque viruses being the predominant population. The significance of having two phenotypes of CHIKV circulating in nature is unclear. However, we hypothesized that this may be beneficial to the virus, because to maintain its viability, CHIKV must have a host-switching mode during its life cycle and therefore each phenotype may have a specific host preference. This point requires further investigation.

There are several factors that can mediate plaque size. One is genetic change. An association between gene mutations and plaque variants was reported for CHIKV isolated from the Comoros Islands [27]. The plaque phenotypes of this isolate are determined by two aa substitutions. One is located in the nsP2 region, where the large-plaque virus has a tyrosine (Y642) and the small-plaque virus has a cysteine (C642). The other is a substitution at position 524 in nsP3. The large-plaque virus has an arginine (R524), while the small-plaque variant has a stop codon (X524). Another example of the role of genetic change in plaque phenotype was presented by Lim and colleagues [28], who found that a large-plaque CHIKV exhibited four unique aa substitutions in nsP1, nsP2 and 6K, while a small-plaque variant had three substitutions in nsP1 and E2. In our study, the genome sequencing of small- and large-plaque viruses revealed nine aa changes. When the aa sequences were compared to those of 23 different CHIKV isolates, 8 aa changes were noted. For CHIKV-033L these changes were in nsP1 (S29 and P407), nsP2 (M675) and E2 (S384), while for CHIKV-033S the changes were in nsP1 (N146) and nsP3 (P79, I303 and P354) (Table 3). In addition, there is also the deletion of two aa in CHIKV-003L and the presence of a stop codon in CHIKV-033S, both of which are located at the end of nsP3. Either an individual difference or a combination of these sequence differences in CHIKV-033L and CHIKV-033S may contribute to the plaque size.

Investigation of the replication efficiency of HFLS revealed that the small-plaque variants replicated more rapidly and to higher titres than the large-plaque viruses and the parental clinical isolates. This is in agreement with reports based on CHIKVs isolated from the Comoros Islands and Sri Lanka [27, 28]. Interestingly however, small-plaque variants caused more rapid CPE than the large-plaque viruses. This contradicts the general understanding that large-plaque variants typically replicate faster, give a higher virus yield and cause more severe CPE, while small-plaque viruses tend to be less virulent [29–32]. However, there are some exceptions. For example, a study by Wu and colleagues showed that the small plaque size of Japanese encephalitis

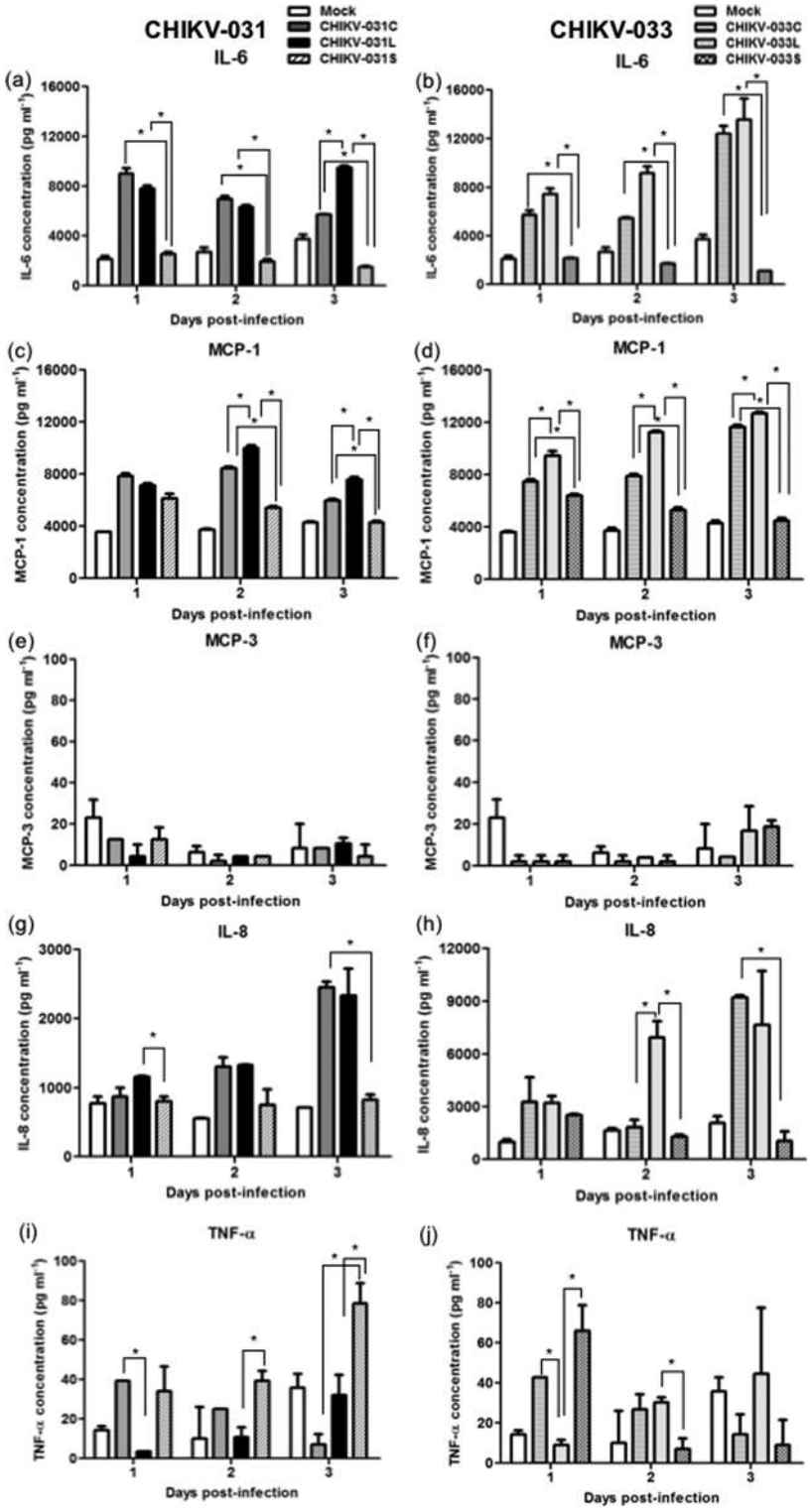

Fig. 3. Selection of cytokine and chemokine production from HFLS infected with clinical isolates, large-plaque CHIKV and small-plaque CHIKV. Cultures were infected at an m.o.i. of 0.01 and supernatants were harvested for 3 constitutive days. The amount of (a, b) IL-6, (c, d) IL-8, (e, f) MCP-1, (g, h) MCP-3 and (i, j) TNF-α production was quantitated by ELISA. The data show the mean±SD from three independent experiments. The data were assessed statistically using Student's *t*-test with *P* value<0.05 (*).

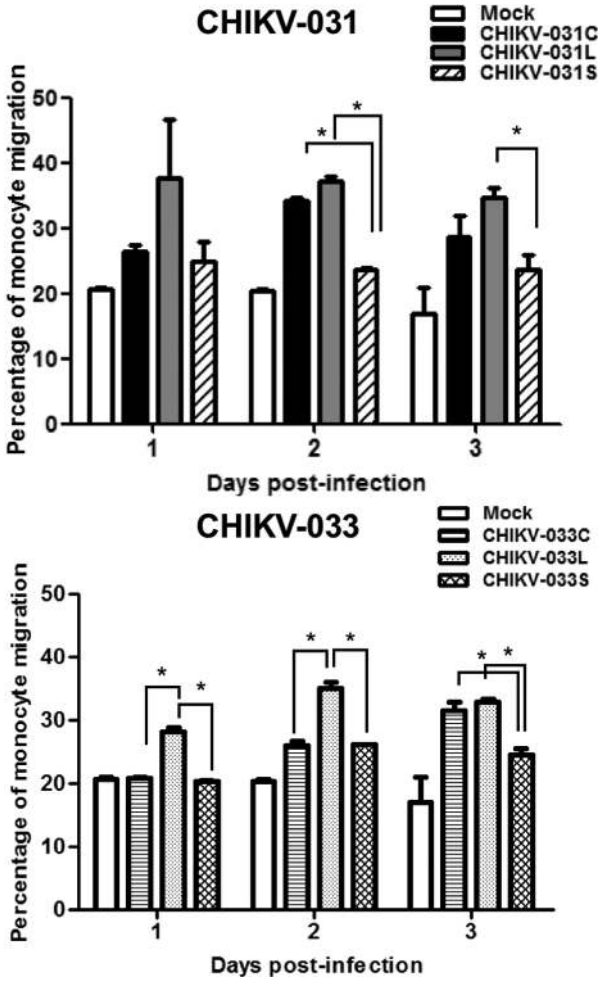

Fig. 4. Different percentages of monocyte migration observation for monocytes culturing with supernatants from HFLS infected with CHIKVs. A monocyte migration assay was performed by adding 2×10^5 primary human $CD14^+$ monocytes in the apical chamber of a trans-well. The media from infected HFLS and mock-treated cells were added to the basal chamber. At 14 h after incubation at 37 °C, 5 % CO_2, the number of migrating cells was determined and expressed as a percentage. The data were assessed statistically using Student's *t*-test with *P* value <0.05 (*).

virus is not attenuated and is neuroinvasive and neurovirulent in mice, whereas the large-plaque variant is attenuated and exhibits reduced mouse neurovirulence [33]. Similarly, a study of a small-plaque variant of Ross River virus (RRV) infection in mice reported enhanced disease severity and mortality as compared to infection with wild-type RRV [34]. In addition, small-plaque mutants of influenza virus showed increased virulence in mice and were less sensitive to neutralization by murine collectins as compared to the wild-type virus [35]. These adaptations and mutations of the viruses are beneficial for increasing infectivity and fitness. In this regard, the most relevant differences between CHIKV-033L and CHIKV-033S that might contribute to

Table 2. Synonymous nucleotide differences in CHIKV-033S and CHIKV-033L

	CHIKV-033S	CHIKV-033L	Nucleotide position	Amino acid position	Location in genome
1	GA**C**	GA**T**	301	76	nsP1
2	GT**A**	GT**G**	2089	671	nsP2
3	CT**G**	CT**A**	2131	685	nsP2
4	TT**G**	TT**A**	2149	691	nsP2
5	TC**T**	TC**A**	2251	719	nsP2
6	**T**TG	**C**TG	2780	896	nsP2
7	TG**C**	TG**T**	4255	1393	nsP3
8	TA**C**	TA**T**	4675	1533	nsP3
9	TT**G**	TT**A**	6319	2081	nsP4
10	GG**G**	GG**T**	7348	2424	nsP4
11	TC**T**	TC**C**	7381	2435	nsP4
12	TT**C**	TT**T**	7432	2452	nsP4
13	AA**G**	AA**A**	7821	86	Capsid
14	TT**C**	TT**T**	9096	511	E2
15	TG**C**	TG**T**	9216	551	E2
16	AC**G**	AC**A**	9420	619	E2
17	AA**C**	AA**T**	10 293	910	E3
18	G	A	11 330		3′ UTR
19	C	T	11 421		3′ UTR
20	C	T	11 426		3′ UTR
21	T	C	11 476		3′ UTR
23	A	G	11 493		3′ UTR

the differences in infectivity, replication and host cell responses are the deletion of two aa or the presence of a stop codon at the end of nsP3 (CHIKV-033L and CHIKV-033S, respectively). Interestingly, the deletion of two aa seen in CHIKV-033L was not observed in the 23 other CHIKV isolates. The stop codon present in the nsP3 in CHIKV-033S is common among alphaviruses and other CHIKV clinical isolates. The opal termination codon in nsP3 results in the translation of two different polyproteins, P123 and P1234. Approximately 90 % of synthesized polyprotein is P123 and only a small amount of P1234 is synthesized [36, 37]. In CHIKV-033L, which lacks the stop codon, only P1234 polyprotein is synthesized. For other alphaviruses, such as Semliki Forest virus (SFV) and o'nyong nyong virus (ONNV), it has been shown that for some isolates the stop codon is replaced with arginine. For ONNV the stop codon is associated with higher infectivity and replication kinetics [38], which is consistent with CHIKV-033S's higher infectivity and replication (Fig. 2c). In SFV the presence of stop codon was shown to be related to the pathogenicity of the virus [39]. It is possible that either one difference or a combination of these differences could influence virus phenotype and contribute to the differences between CHIKV-033S and CHIKV-033L. Our results suggest that CHIKV-031S and CHIKV-033S may have a growth advantage in HFLS over that seen in Vero cells, and vice versa for CHIKV-031L and CHIKV-033L. Which factor or factors

Table 3. Non-synonymous nucleotide changes in CHIKV-033S and CHIKV-033L

	CHIKV-033S	aa	CHIKV-033L	aa	Nucleotide position	Amino acid position	Location in genome
1	CCA	Proline	TCA	Serine	161	29	nsP1
2	AAC	Asparagine	GAC	Aspartic acid	512	146	nsP1
3	AAT	Asparagine	GAT	Aspartic acid	632	186	nsP1
4	CTC	Leucine	CCC	Proline	1296	407	nsP1
5	ACG	Threonine	ATG	Methionine	3705	1210 (675)	nsP2
6	CCT	Proline	TCT	Serine	4310	1412 (79)	nsP3
7	ATC	Isoleucine	GTC	Valine	4982	1636 (303)	nsP3
8	CCG	Proline	CTG	Leucine	5136	1687 (354)	nsP3
9	TTA	Leucine	–	–	5642–5645	1856 (523)	nsP3
10	TGA	Stop	–	–	5645–5647	1857 (524)	nsP3
11	GGT	Glycine	AGT	Serine	9688	709 (384)	E2

determine the growth advantage in HFLS is under investigation.

Fibroblast-like synoviocytes, the major population of the synovium, play a key role in inflammatory cascade complications and are known to be central mediators of joint damage in inflammatory arthritides of either an infectious or a non-infectious origin [40], and the synovium has been shown to be a target of CHIKV infection [19]. Therefore, examining the responses of fibroblast-like synoviocytes against the large- and small-plaque variants should advance our understanding of the pathogenic role of these variants in CHIKV-induced arthralgia/arthritis. Several inflammatory cytokines/chemokines are known to participate in the pathogenesis of CHIKV-induced arthralgia/arthritis [12], and reports have demonstrated a link between IL-1β and IL-6 and severity during the acute phase of CHIKF, while elevation of IL-6, MCP-1 and IL-8 is associated with the chronic phase or prolonged arthralgia [19, 41, 42]. Among these mediators, IL-6 may serve as a principal mediator for inflammation, pain and bone lost during arthralgia/arthritis caused by alphavirus infection [12, 43, 44], as IL-6 is upregulated in the affected joints and blocking IL-6 activity decreases the disease severity [45, 46].

IL-8 is one of the chemokines that participates in bone loss, pain and inflammation in rheumatoid arthritis [47, 48]. IL-8 can drive macrophages into osteoclasts via an autocrine loop. Moreover, osteoclast-derived IL-8 can induce nociceptive pain [49, 50]. In addition, IL-8 induces migration of neutrophils to affected joints and the recruited neutrophils can contribute to the generation of inflammatory arthritis via the lipid–cytokine–chemokine cascade [51].

Beside soluble mediators, immune cells such as monocyte/macrophage have been shown to participate in arthritogenic alphavirus infection [25, 44, 52]. Blocking these inflammatory cells ameliorated alphavirus-induced arthritis and myositis in a mouse model [53]. For our present work, we showed that the supernatant of HFLS infected with the large-plaque variants or the respective parental clinical isolates contained a high level of MCP-1 and exerted strong chemotactic activities, while the small-plaque viruses were only able to migrate monocytes at a level comparable to the supernatant from mock-infected HFLS.

Our investigation showed that CHIKV-031L, CHIKV-033L and the parental CHIKVs upregulated the production of IL-6, IL-8 and MCP-1 in infected HFLS and strongly induced the migration of primary human monocytes/macrophages. These results suggest that the large-plaque viruses are crucial for arthralgia/arthritis development. The role of small-plaque viruses in the pathology of the joints is unclear, however, we hypothesize that the small-plaque virus may contribute to a certain degree towards the joint pathology, as infection by this variant induced rapid and severe damage of HFLS.

In conclusion, we investigated, for the first time, the interaction between HFLS and the large- and small-plaque variants of CHIKV that were obtained directly from patients' samples. Our results suggest a potential role of large-plaque viruses in joint inflammation and arthralgia/arthritis formation, while small-plaque variants may contribute to virus-induced tissue damage. Why the circulating CHIKV harbours different phenotypes is unclear, but is clearly of great interest. Future studies using infectious clone and site-directed mutagenesis will identify key determinants that contribute to the CHIKV-033S and CHIKV-033L phenotypes.

METHODS

Cell culture

Aedes albopictus (C6/36) cells were cultured in minimal essential medium (MEM) (Gibco, Thermo Scientific, MA, USA) with 10 % heat-inactivated foetal bovine serum (FBS) (Gibco, Thermo Scientific) at 28 °C and 5 % CO_2 in a humidified incubator. Vero cells (African green monkey kidney epithelial cells) were cultured in M199 medium (Gibco, Thermo Scientific) with 10 % FBS (Gibco, Thermo Scientific) at 37 °C and 5 % CO_2 in a humidified incubator. Human fibroblast-like synoviocyte (HFLS) cells were purchased from Cell Applications, Inc. (San Diego, CA, USA). The cells were isolated from the synovium of a healthy 60year-old male according to the manufacturer's instructions. The synoviocytes were

characterized by a fibroblast-like morphology and growth pattern, and the expression of CD55 on the cell surface. The cells were cultured in synoviocyte growth medium supplemented according to manufacturer's protocol (415–500; Cell Applications, CA, USA) at 37 °C and 5 % CO_2 in a humidified incubator. The culture medium was changed every alternate day, and the cells were sub-cultured when they reached 80–90 % confluence.

Virus stock and plaque purification

The chikungunya viruses used in this study were isolated from patients who presented with severe CHIKF at the Community Medical Unit, Pang Nga Hospital, Pang Nga, Thailand, as previously reported [24]. Two clinical isolates, CHIKV-031C and CHIKV-033C, were used in the present study. The viruses were isolated from the patients' sera using C6/36 cells [25]. The working seeds in this study were viruses from the second passage. The CHIKV titres were quantified using a plaque assay on Vero cells.

The isolation of large- and small-plaque phenotypes of CHIKV was performed by plaque purification as described by others [54]. Briefly, Vero cells were seeded at 2×10^5 cell ml^{-1} in six-well tissue culture plates and cultured for 2 days. The viruses were serially diluted to dilutions that produced between three and five well-separated plaques per well. The diluted viruses were inoculated on a Vero cell monolayer for 90 min and then the cells were washed three times with 1×PBS to remove the unbound viruses. The inoculated cells were overlaid with overlay medium containing Earle's balanced salt solution, lactalbumin (US Biological, MN, USA), yeast extract (BD Bioscience, MD, USA), heat-inactivated foetal bovine serum and 1 % agarose (Gibco, Thermo Scientific) before being incubated at 37 °C and 5 % CO_2 for 6 days. The second overlay was subsequently performed using 0.05 % neutral red (Sigma-Aldrich, USA) in 1 % agarose (Gibco, Thermo Scientific). The clearly visible plaques were marked and gently picked using a p200 pipette with a filter tip. The agarose plug contained in the tip was immediately transferred to the culture of C6/36 cells (one plug/well). The inoculated C6/36 cultures were cultured for 4 days. The supernatants of infected cultures were harvested and plaque purification was repeated until homogeneous large- or homogeneous small-plaque viruses were obtained. The obtained large- and small-plaque viruses were amplified once in C6/36 cells before being used as working seeds.

Infection of HFLS cultures

Infections of HFLS were performed as previously described [25]. Briefly, HFLS cells were seeded into 12-well plates at 8×10^4 cells/well and cultured for 24 h. The monolayers of cells were washed once with medium without FBS, and the cells were subsequently incubated with 200 µl of CHIKV (clinical isolates, large- and small-plaque variants of CHIKV) at an m.o.i. of 0.01 p.f.u. $cell^{-1}$ at 37 °C for 90 min. The inoculated cultures were then washed three times with 1×PBS to remove unbound viruses. The cultures were maintained in 1.5 ml of complete culture medium with the manufacturer's recommended supplements at 37 °C in the presence of 5 % CO_2. Two hundred microlitres of the culture supernatants were harvested every 24 h for 5 consecutive days, and the cultures were replenished with the same volume of fresh synoviocyte growth medium. The harvested supernatants were clarified for cell debris by centrifugation at 1500 r.p.m. at 4 °C for 10 min. The cleared supernatants were quantified for viral production by plaque assay, with mock-infected HFLS culture being used as a negative control.

Plaque assay

Briefly, Vero cells were seeded into 12-well plates at a density of 1×10^5 cells per well and cultured to confluence for 2 days. Subsequently, 200 µl of serially 10-fold diluted samples were added to the monolayer and the cells were incubated at 37 °C in 5 % CO_2 for 90 min with occasional agitation. After adsorption and penetration, the monolayer of the cells was covered with plaque nutrient containing 1 % agarose and then incubated at 37 °C and 5 % CO_2 for 7 days. The cells were fixed with 3.7 % formalin in PBS and stained with 12.5 % (W/V) crystal violet. Viral titres were determined as p.f.u.ml^{-1} [55]. Plaque morphology was observed and the diameter of the plaques was measured.

Detection of cytokines and chemokines production by ELISA

The supernatants from CHIKV-infected and diluent-treated control cultures were quantified for TNF-α, IL-6, IL-8, MCP-1 and MCP-3. All of the ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). All ELISAs were performed according to the procedure supplied by the manufacturer.

Migration assay

Cell migration assays were performed according to previously described procedures with minor modifications [56]. The assay was performed using a 24-well transwell inserted with 8 µm-pore-diameter polycarbonate filters (Corning, Corning, NY, USA). The $CD14^+$ human primary monocytes were isolated from a healthy donor as previously described [25]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from 50 ml peripheral blood using Lymphoprep (Axis-Shield PoCAS, Oslo, Norway). The monocytes were purified from PBMCs using an anti-CD14 antibody and magnetic bead isolation (MACS, Miltenyi Biotec, Germany). A total of 10^5 purified monocytes in 200 µl were seeded into the upper chamber and the same volume of cultured supernatants from CHIKV-infected HFLS was added to the basal chamber. The number of monocyte cells that migrated from the upper chamber was counted after 14 h of incubation at 37 °C, 5 % CO_2 in a humidified incubator. Non-migrating cells were detached with a cotton swab and the membranes were removed, fixed with methanol and then stained with eosin and methylene blue. The number of cells that were attached to the transwell membranes was

determined. The number of cells that migrated was expressed as a percentage of the total number of cells.

Sequencing and sequence analyses of plaque purified virus

Total RNA from C6/36 cells infected with CHIKV-033S or CHIKV-033L was extracted using Trizol (Thermo Fisher Scientific, MA, USA). One microgram of total RNA was reverse-transcribed using random nonamer primers and MMLV reverse transcriptase (Sigma-Aldrich, Inc., USA) according to the manufacturer's instructions. cDNA was used to amplify 1200–1700 bp-long PCR fragments using Phusion High-Fidelity DNA polymerase (New England Biolabs, Inc., USA). The PCR primers used are listed in Table S1 (available in the online version of this article). Sequencing was carried out by the Australian Genome Research Facility (AGRF, Brisbane, Australia). DNAStar (SeqManPro and MegaAlignPro) and BioEdit software was used to analyse the viral sequences.

Statistical analysis

Statistical analysis was performed using the GraphPad prism program, version 5 (GraphPad software, Inc., La Jolla, CA, USA). For comparison between two study groups, Student's *t*-test was used with $P<0.05$ (*) being considered a significant difference.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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