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# The synergistic effect of nsP2-L$^{618}$, nsP3-R$^{117}$, and E2-K$^{187}$ on the large plaque phenotype of chikungunya virus

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## Abstract

Chikungunya virus (CHIKV), a mosquito-borne *Alphavirus*, is the etiological agent of chikungunya fever. CHIKV re-emerged from 2004 onwards, and subsequently caused major outbreaks in many parts of the world including the Indian Ocean islands, Asia, and the Americas. In this study, a large plaque variant of CHIKV isolated from patient in Thailand was subjected to repeated cycles of plaque-purification in Vero cells. The resulting virus produced homogenous large plaques and showed a more pathogenic phenotype than the parental wild-type CHIKV. Whole genome analysis of the large plaque virus in comparison to parental isolate revealed a number of mutations, leading to the following amino acid changes: nsP2 (P$^{618}$→L), nsP3 (G$^{117}$→R), and E2 (N$^{187}$→K). Eight recombinant CHIKVs were constructed to determine which amino acids mediated the large plaque phenotype. The results showed the recombinant virus which contains all three mutations, rCHK-L, produced significantly larger plaques than the other recombinant viruses ($p < 0.01$). Moreover, the plaque size of the other recombinant virus tended to be smaller if they contained only one or two of the large plaque associated mutations in the viral genome. In conclusion, the combination of all three residues (nsP2-L$^{618}$, nsP3-R$^{117}$, and E2-K$^{187}$) is required to produce the large plaque phenotype of CHIKV.

**Keywords** Chikungunya virus · Plaque morphology · Plaque-purification · Large plaque-associated mutations

## Introduction

Chikungunya virus (CHIKV), a re-emerging arbovirus, is the causative agent of chikungunya fever, a dengue-like illness characterized by a high fever, skin rash, myalgia, and/or arthralgia. Notably, the arthralgia can be prolonged,

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persisting for months or years in some cases. The name "Chikungunya" comes from the Makonde language and means "that which bends up" reflective the arthritic symptoms of the disease [1, 2]. CHIKV was first isolated in Africa in 1955 and became epidemic periodically. Later on, the virus spread from Africa to Asia including Thailand where the first Asian case was reported in 1958 [3, 4]. Despite a long history of the disease, chikungunya only came to prominence internationally after a massive outbreak in 2005–2006 in La Reunion Island in which approximately 270,000 cases were reported, followed by over a million cases in Asia over the subsequent years [5–7]. Subsequently, CHIKV emerged in the Americas where autochthonous transmission was reported for the first time in December 2013 [8, 9].

CHIKV is a member of the genus *Alphavirus* in the family *Togaviridae*. It is a small spherical enveloped virus with a linear positive sense single stranded RNA genome of approximately 11.8 kilobases (kb). The CHIKV genome has a 5′-7-methylguanosine cap and a 3′ poly A tail flanking 2 open reading frames encoding for nonstructural and structural polyproteins [10, 11]. The nonstructural proteins

(nsP1, 2, 3, and 4) are essential for the viral transcription/replication processes, with nsP1 mediating viral RNA capping and nsP2 functioning as a helicase and as a protease to cleave the polyprotein into mature proteins. Of the remaining nonstructural proteins, nsP3 is required as part of the viral replicase complex and is a regulator of the cellular stress response, while nsP4 is a RNA-dependent RNA polymerase [10, 12–14]. The structural proteins, which are translated from a 26S subgenomic mRNA, consist of the capsid protein, two envelope glycoproteins (E1 and E2) and two additional small peptides (E3 and 6k) [10, 11]. Based on phylogenetic studies of the E1 protein, CHIKV was grouped into three distinct lineages, the West African, the Asian and the East, Central and South African (ECSA) lineages that were designated according to their historic transmission areas. However, this does not represent the recent transmission areas of CHIKV, as the ECSA lineage caused infections in a large part of Asia, while the Asian lineage was introduced into the Americas where it continues to circulate [6, 15–18].

Plaque morphology is a good surrogate for viral fitness as it reflects viral replication and cell-to-cell spread in a particular cell type [19, 20]. In common with other RNA viruses, the CHIKV RNA-dependent-RNA polymerase lacks proof reading activity, resulting in extremely high mutation rates [21]. As a result, these viruses exist as mixed populations of virus variants, which can be observed as plaque size heterogeneity [22–24]. This phenomenon was seen in our primary isolates of CHIKV from Thai patients and this study sought to understand the genetics and virulence of the large plaque viral variant. We placed selective pressure on the parental virus isolate via propagation and selection through Vero cells, resulting in a CHIKV that produces homogenous large plaques, and comparatively characterized the large plaque-size variant with the wild-type CHIKV. Furthermore, the genetic sequences responsible for large plaque phenotype were determined and confirmed by reverse genetics.

## Materials and methods

### Cells and viruses

C6/36 cells, which are derived from *Aedes albopictus* larvae cells, were maintained at 28 °C in minimum essential medium (MEM; Gibco, CA, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS; HyClone, UK) and 100 units of penicillin/streptomycin per ml. Vero cells, derived from African green monkey kidney epithelial cells, were maintained at 37 °C, 5% $CO_2$ in Dulbecco's modified Eagles medium (DMEM) supplemented with 5% FBS and 100 unit of penicillin/streptomycin per ml.

CHIKV was isolated from a patient in Phang-nga province, Thailand in 2009 and passaged two times in C6/36

cells. The virus was designated as WT-CHK025. CHIKV stock was propagated in C6/36 cells. The culture medium of the infected cells was harvested at 40 h after infection and stored at − 80°C until use.

### Standard plaque assay

Viruses were tenfold serially diluted in BA-1 medium (1xM-199E, 1 M Tris–HCl pH 7.6, 2% (w/v) BSA, 100 units of penicillin/streptomycin per ml, 0.075% (w/v) $NaHCO_3$) and added to monolayers of Vero cells in 6-well plates. The cells were incubated for 2 h at 37 °C and overlaid with nutrient agarose [0.8% agarose (SeaKem LE, USA) containing Earle's balanced salts supplemented with 0.5% (w/v) yeast extract, 2.5% lactalbumin hydrolysate, 3% FBS] and incubated at 37 °C, 5% $CO_2$ for 4 days. For plaque-purification, the cell layer was stained using a second overlay containing 1% neutral red and incubated as above for 16-18 h. To determine virus titers or plaque size, after 5 days of infection, Vero cells were fixed with 8% formaldehyde in PBS at room temperature for 2 h, the solid media were removed and the plaques were observed using methylene blue staining. Thirty plaques of each virus were measured for diameter in millimeters and the plaque sizes were compared for statistical significance using analysis of variance (ANOVA) (GraphPad software).

### Selection and purification of large plaque virus

WT-CHK025 at passage 5 was used in plaque assay and large plaques from the terminal dilution were picked using sterile pipette tips, suspended in serum-free medium and inoculated directly onto fresh Vero cells. After cytopathic effect (CPE) was observed, the viral supernatant was harvested and amplified in Vero cells before being subjected to further plaque-purification. The plaque selection and purification processes were continued until passage 17 at which stage homogenous large plaques were observed. The virus was designated as CHK-L.

### Viral pathogenicity in suckling mice

Pregnant ICR mice were purchased from the National Laboratory Animal Center (Mahidol University, Thailand) and were subsequently housed in the Institute of Molecular Biosciences (Mahidol University, Thailand) animal facility. Groups of 50 and 45 suckling mice were intracranially injected (i.c.) with $10^3$ pfu of WT-CHK025 or CHK-L, respectively. The mice were observed daily for any sign of sickness for 21 days. Moribund mice were euthanized and the results were analyzed for significance in a Kaplan–Meier survival plot using the Prism software (GraphPad software). The animal experiment was performed under a protocol

approved by the Institutional Animal Care and Use Committee (#MB-ACUC 2016/006).

## cDNA synthesis, PCR, sequencing and phylogenetic analysis

Total RNA was extracted from the supernatant or cell lysate of WT-CHK025 passage 2-infected cells using Trizol reagent (Invitrogen, USA.) following the manufacturer's protocol. One μg of RNA was reverse transcribed using Improm II™ reverse transcriptase with random hexamers or oligo-dT being used as the primer (Promega, USA.).

To design specific primers for PCR, several sequences of CHIKV isolates were aligned to obtain a consensus sequence using ClustalW web online. Nine primer pairs were designed to amplify 9 overlapping fragments covering the entire genome of CHIKV using ApE (Davis http://www.biology.utah.edu/jorgensen/wayned/ape/) and Primers3 (Lincoln et al. http://frodo.wi.mit.edu/primer3/) Softwares. The sequences of the primers can be found in the supplementary document. Some primers were synthesized based on Sreekumar et al. [7]. The cDNA was amplified using Platinum@ Taq DNA polymerase (Invitrogen, USA). The 5′ end of the viral RNA was amplified using a 5′/3′ RACE, 2nd Generation Kit (Roche, Switzerland) following the manufacturer's instruction. The PCR products were purified using a Gel/PCR DNA fragments extraction kit (Geneaid, Taiwan).

Each purified PCR fragment was cloned into the sequencing vector, p-SC-A vector (Takara, Japan) or pCR@2.1vector (Invitrogen, USA.). Three clones of recombinant plasmids for each fragment were sequenced using two universal primers (M13F and M13R) and one specific internal primer. The sequences were analyzed to reconstruct the whole WT-CHK025 genome using BioEdit Sequence Alignment Editor Program (version 7.0.5.3). The whole genome sequence was subjected to phylogenetic analysis using the neighbor-joining method calculated with the CLC Sequence Viewer 6.6.1 software.

The whole genome sequence of CHK-L was obtained from three clones of virus from the same passage. RNA extraction, cDNA synthesis, and PCR were performed using the same procedures as for CHK-WT025. PCR products were directly sequenced and the sequencing results were aligned to determine consensus sequence which was compared to the WT-CHK-025 sequence using BioEdit.

## Construction of full-length cDNA clones of CHK-025 and large plaque variant

First strand cDNA was used as a template to synthesize 5 overlapping fragments using specific primer pairs (the sequences are available in the supplementary document) and Phusion high-fidelity DNA Polymerase (Invitrogen,

USA). The fragments were sequentially cloned into pBluescript KS(+) plasmid vector (Strategene, USA) using unique restriction sites (*Sac*I, *Sma*I, *Age*I, *Cla*I, *Xho*I, and *Apa*I). A T7 RNA polymerase promoter and *Psp*OMI recongnition site were introduced 5′-upstream and 3′-downstream of the genome, respectively. The recombinant plasmid containing a full-length cDNA of CHIKV was designated as pCHK-WT.

To generate the full-length infectious CHIKV clones with large plaque associated mutations, the first cDNA fragment containing nsP2-618 and nsP3-117 and the second fragment containing E2-187 were cloned into pBluescriptKS(+) plasmids for use as templates for mutagenesis. Site-directed mutagenesis was used to generate amino acid substitution nsP2 ($P^{618} \rightarrow L$) and nsP3 ($G^{117} \rightarrow R$) in the first plasmid, and E2 ($N^{187} \rightarrow K$) in the second plasmid. The two mutated fragments were exchanged with the corresponding regions of pCHK-WT using *Fsp*AI, *San*DI and *Cla*I, *Xho*I digestions, respectively, resulting in the pCHK-L plasmid cDNA clone. A variety of full-length cDNA clones were created by generating different combinations of large plaque-associated mutations resulting in 6 plasmids; pCHK-L(AB) containing nsP2 ($P^{618} \rightarrow L$) and nsP3 ($G^{117} \rightarrow R$), pCHK-L(AC) containing nsP2 ($P^{618} \rightarrow L$) and E2 ($N^{187} \rightarrow K$), pCHK-L(BC) containing nsP3 ($G^{117} \rightarrow R$) and E2 ($N^{187} \rightarrow K$), pCHK-L(A) containing nsP2 ($P^{618} \rightarrow L$), pCHK-L(B) containing nsP3 ($G^{117} \rightarrow R$), and pCHK-L(C) containing E2 ($N^{187} \rightarrow K$).

## Recovery of the recombinant viruses

Full-length cDNA plasmids were linearized using the *Psp*OMI restriction enzyme (New England BioLab, USA) and in vitro transcribed using a T7 High Yield RNA synthesis Kit (New England BioLab, USA) at 16 °C for overnight. The transcribed RNA was transfected into C6/36 cells using Lipofectamine2000® (Invitrogen, USA) and the cells were incubated for 5 days. To examine the recovered virus, supernatants were transferred to Vero cells for observation of CPE and plaque assay. The recovered viruses were propagated in C6/36 cells and the mutations were confirmed by sequencing.

## Results

### Generation of a large plaque variant of CHIKV

CHIKV primary isolates show plaque size heterogeneity (Fig. 1a–c). To obtain a virus that produced uniform large plaques, passage 5 of the CHIKV isolate 025, originally isolated from the serum of Thai patient (WT-CHK025) was used for large plaque-purification and propagation in Vero cells. After 2 and 3 large plaque-purification cycles in Vero cells, virus passage 9 (Fig. 1e) and passage 11 (Fig. 1f)
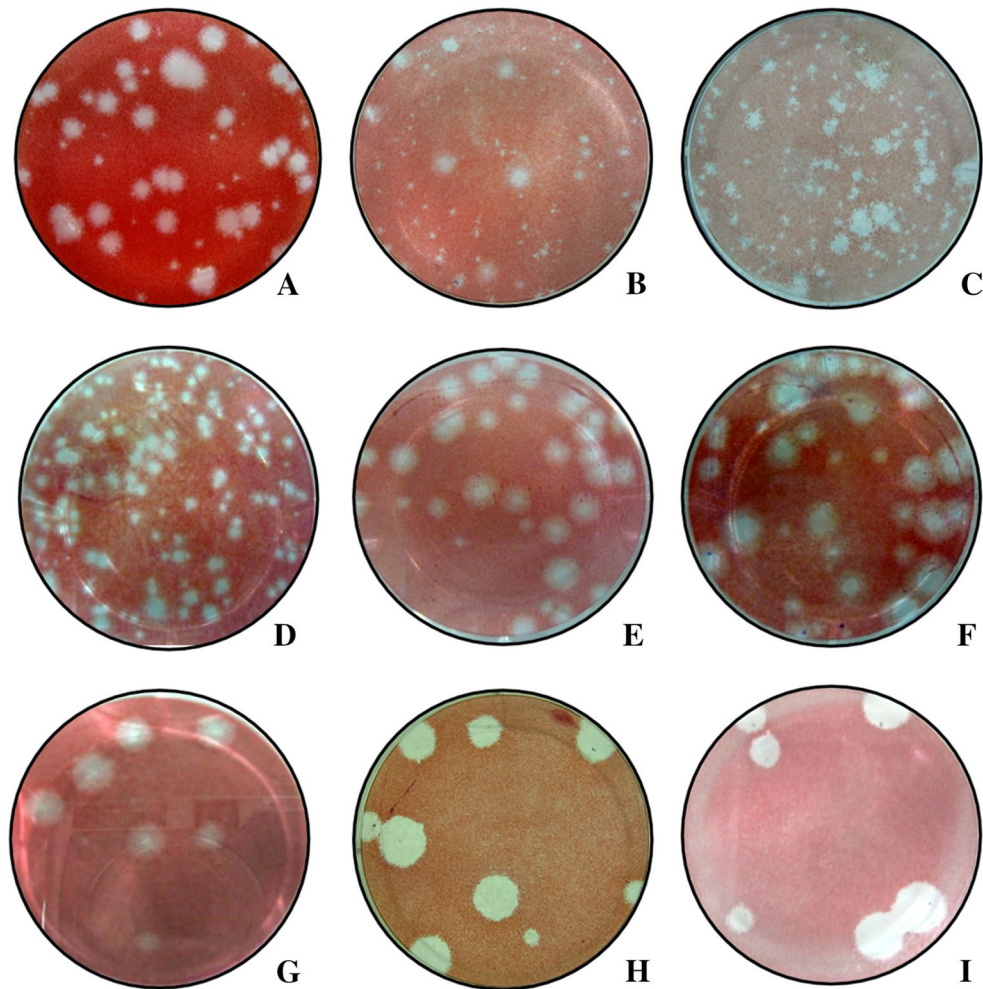
**Fig. 1** Plaque morphologies of CHIKV isolates (**a–c**) and CHIKVs during the large plaque selection process (**d–i**). WT-CHK025 (**c**) underwent propagation and plaque-purification in Vero cells until passage 17 (**i**), which showed a uniform large plaque morphology, designated as CHK-L. **d–i** are plaques of viruses from passage 7, 9, 11, 13, 15, and 17, respectively. Standard plaque assays were undertaken using Vero cells with 5 days of incubation. Each circle represents one well of a 6-well plate

still showed a heterogenous plaque morphology. However, following a total of 12 passages, alternating with 5 large plaque-purifications, the resulting CHIKV produced homogenous large plaques. This virus was designated as CHK-L (Fig. 1i).

## Pathogenic characterization of wild-type and large plaque CHIKVs

Plaque size often correlates with the pathogenicity of the virus. Frequently, viruses that produce small plaques are less pathogenic than their variants that produce larger plaques [20, 25]. Therefore, we hypothesized that our large plaque CHIKV, CHK-L, should be more pathogenic than the original wild-type virus. The pathogenicity of the wild-type and large plaque CHIKVs were compared by

a mouse-neurovirulence study in suckling mice. Groups of newborn mice were intracranially injected with $10^3$ pfu of WT-CHK025 or CHK-L. The mice were kept for observation for 3 weeks and the numbers of surviving mice were used to plot survival curves (Fig. 2). Mice that received CHK-L succumbed to the infection from 4 dpi onwards and only 17.8% of the cohort survived at the end of the experiment. Despite the fact that the WT-CHK025 inoculated group started showing symptoms of the disease only one day later than the CHK-L inoculated group, mice that received WT-CHK025 showed a survival rate of 74% at 21 dpi. This result reflects the correlation between CHIKV plaque size and pathogenicity in which the virus that produced larger plaques was more pathogenic than the parental virus that produced mixed-size plaques.
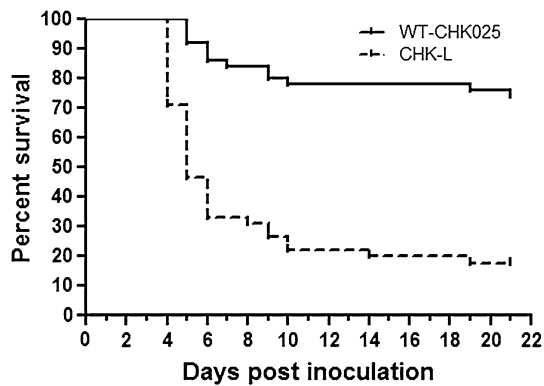
**Fig. 2** Neurovirulence of wild-type and large plaque CHIKVs. Groups of suckling mice were intracranially inoculated with $10^3$ pfu of WT-CHK025 (solid line) or CHK-L (dashed line). Mice were daily observed for sign of sickness and survival was recorded for 21 days. The Kaplan–Meier plot shows the percentage of surviving mice on each day

## Genotypic characterization of wild-type and large plaque CHIKVs

The size of the entire genome of WT-CHK025 is 11,811 bp with a 5′ UTR and a 3′ UTR of 76 and 498 nucleotides, respectively. The length of the first open reading frame (ORF) is 7422 nucleotides while the second ORF is 3747 nucleotides. The genome organization is identical to that of the La Reunion 2006 isolate, CHIKV LR-2006. The result of phylogenetic analysis based upon the whole genome sequence using the neighbor-joining method showed that WT-CHK025 is classified in the ECSA lineage (Fig. S1). The sequence of WT-CHK025 is available in GenBank with the accession number KP164869.

The whole genome of CHK-L was sequenced and compared with that of WT-CHK025 to identify the genetic changes associated with the phenotypes. Six nucleic acid differences between the genome of WT-CHK025 and CHK-L were found at nucleotide positions 1, 476, 3534, 4424, 9102, and 11768, which are located in 5′UTR, nsP1, nsP2, nsP3, E2, and 3′UTR regions, respectively. Four mutations in

the coding region were nonsynonymous. However, after 2 additional clones of large plaque variant were sequenced at those particular positions to verify the differences, only 3 consensus mutations, which lead to 3 amino acid changes at nsP2 ($P^{618}{\rightarrow}L$), nsP3 ($G^{117}{\rightarrow}R$), and E2 ($N^{187}{\rightarrow}K$), were confirmed (Table 1).

## Construction of CHK-025 infectious clone and variety of clones with mutations and characterization of recombinant viruses

To define which of the identified mutations are associated with the large plaque phenotype, we constructed a full-length infectious clone of the WT-CHK025 and incorporated each mutation individually and in combination into this backbone. cDNA fragments containing the whole genome of WT-CHK025 were amplified and sequentially cloned into plasmids using standard restriction enzyme digestion cloning technique, resulting in CHK-WT (Fig. 3).

In order to determine whether the single nonsynonymous mutations at nucleotides 3534, 4424, or 9102 are sufficient to affect the plaque size, site-directed mutagenesis was used to introduce these mutations at those particular positions into the CHK-WT. The resulting constructs were designated as CHK-L(A), CHK-L(B), and CHK-L(C), which contain single amino acid changes in nsP2 ($P^{618}{\rightarrow}L$), nsP3 ($G^{117}{\rightarrow}R$) and E2 ($N^{187}{\rightarrow}K$), respectively. An additional 4 constructs were generated in case that a particular combination of 2 mutations or all three mutations were required for the large plaque phenotype. CHK-L(AB) contains changes in nsP2 ($P^{618}{\rightarrow}L$) and nsP3 ($G^{117}{\rightarrow}R$), CHK-L(AC) contains changes in nsP2 ($P^{618}{\rightarrow}L$) and E2 ($N^{187}{\rightarrow}K$), CHK-L(BC) contains changes in nsP3 ($G^{117}{\rightarrow}R$) and E2 ($N^{187}{\rightarrow}K$) while the construct CHK-L contains all 3 mutations, consistent with the parental CHK-L virus (Fig. 3).

After sequence confirmation, all full-length cDNA clones were used for in vitro transcription and the resulting RNAs were transfected into C6/36 cells. All eight recombinant viruses, rCHK-WT, rCHK-L(A), rCHK-L(B), rCHK-L(C), rCHK-L(AB), rCHK-L(AC), rCHK-L(BC), and rCHK-L,

**Table 1** Comparison of nucleic acid and amino acid sequences of the parental WT-CHK-025 and three clones of CHK-L

| Region | Nucleic acid sequence | | | | Amino acid sequence | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Position | WT-CHK025 | CHK-L #1 | CHK-L #2 | CHK-L #3 | Position | WT-CHK025 | CHK-L #1 | CHK-L #2 | CHK-L #3 |
| 5′UTR | 1 | A | G | A | A | | | | | |
| nsP1 | 476 | T | A | T | T | 134 | C | S | C | C |
| nsP2 | 3534 | C | T | T | T | 618 | P | **L** | **L** | **L** |
| nsP3 | 4424 | G | A | A | A | 117 | G | **R** | **R** | **R** |
| E2 | 9102 | C | A | A | A | 187 | N | **K** | **K** | **K** |
| 3′UTR | 11768 | A | G | A | A | | | | | |

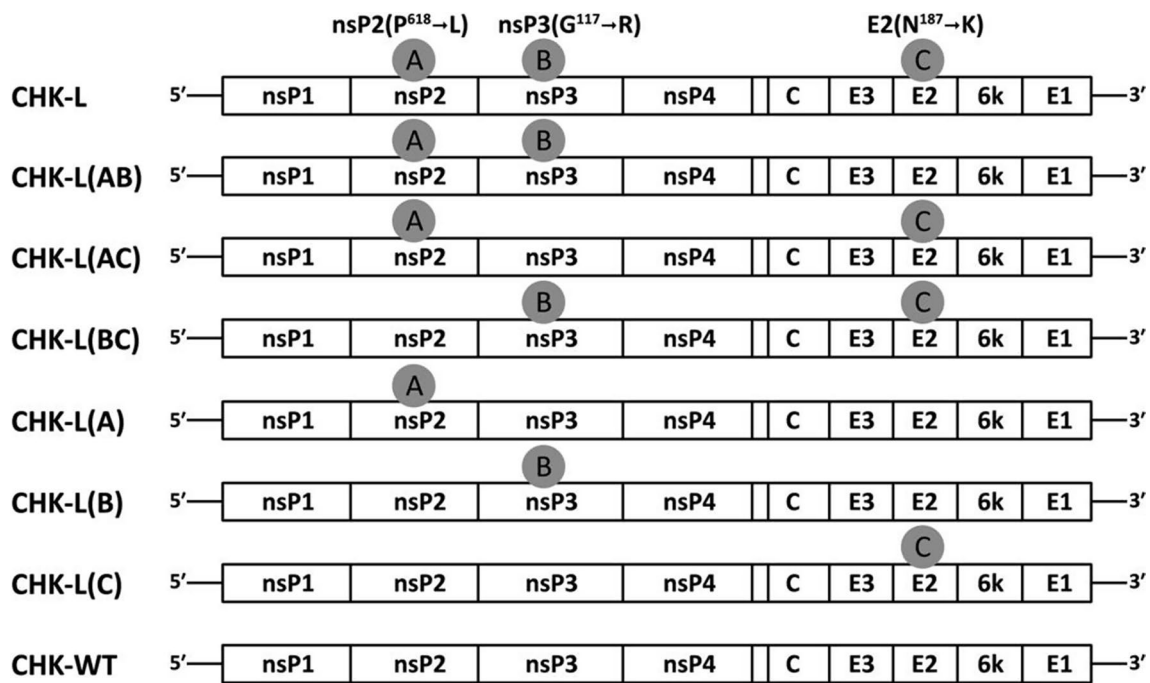The boldfaces show the consensus amino acid changes

**Fig. 3** Schematic representation of the recombinant wild-type and the viruses with different combinations of large plaque-associated mutations. Site-directed mutagenesis was used to generate the nsP2-P618L, nsP3-A117R, and E2-N187K mutations, designated as A, B, and C, respectively. Different combinations of these mutations were introduced into the wild-type infectious clone resulting in seven different infectious clones

were successfully recovered after 5 days of transfection as the supernatants were able to produce CPE in Vero cells (data not shown). The sequences of the recombinant viruses were verified to confirm the introduced mutations and the plaque phenotypes were determined in comparison to the parental CHK-L virus. Figure 4a shows the plaque morphology of the parental virus, and the recombinant CHK-L mutants. It can be seen clearly that the plaques of the recombinant viruses with a single mutation (rCHK-L(A), rCHK-L(B), and rCHK-L(C)), are smaller than those of the recombinant viruses with double or triple mutations. Indeed, statistical analysis showed that the recombinant CHIKVs with a single mutation produced significantly smaller plaques ($p < 0.01$) as compared to recombinant CHIKVs with double or triple mutations or the parental CHK-L virus (Fig. 4b). rCHK-L(A), rCHK-L(B), and rCHK-L(C) generated the plaques with an average diameter of 2.6, 1.8, and 2.5 mm., respectively, which were comparable to the plaque size of the rCHK-WT (1.9 mm.), indicating that the mutations at nsP2 ($P^{618} \rightarrow L$), nsP3 ($G^{117} \rightarrow R$), or E2 ($N^{187} \rightarrow K$) by themselves does not make a significant contribution to CHIKV plaque size ($p > 0.05$).

When a second mutation was added to the constructs, rCHK-L(AB), rCHK-L(AC), and rCHK-L(BC) all displayed a significantly larger plaque sizes (averages of 2.8, 3.0, and 2.7 mm, respectively; $p < 0.01$) as compared to the plaque sizes of rCHK-WT or the recombinant viruses with single mutations. However, the plaques of the double mutation viruses were still smaller than the plaques of rCHK-L, which contains all 3 mutations. Virus rCHK-L displayed the largest plaque size of all of the recombinant viruses, which were larger on average than even the parental CHK-L, suggesting that all 3 residues (nsP2-$L^{618}$, nsP3-$R^{117}$, and E2-$K^{187}$) are required together to generate the large plaque phenotype of CHIKV.

## Discussion

Because of their high mutation rates, which are a consequence of the lack of proof reading activity by the RNA-dependent-RNA polymerases, RNA viruses are known to exist as quasispecies, which is defined as a population of viral variants genetically linked through mutations [26]. These variants compete in equilibrium under certain environmental conditions, and can interact cooperatively on a functional level. Cooperation among viral variants has been shown to be important for successful invasion and pathogenicity for some viruses [27, 28]. The co-existence of the small and large plaque CHIKV variants in nature, as seen with clinical isolates (Fig. 1a–c), suggests that both of them
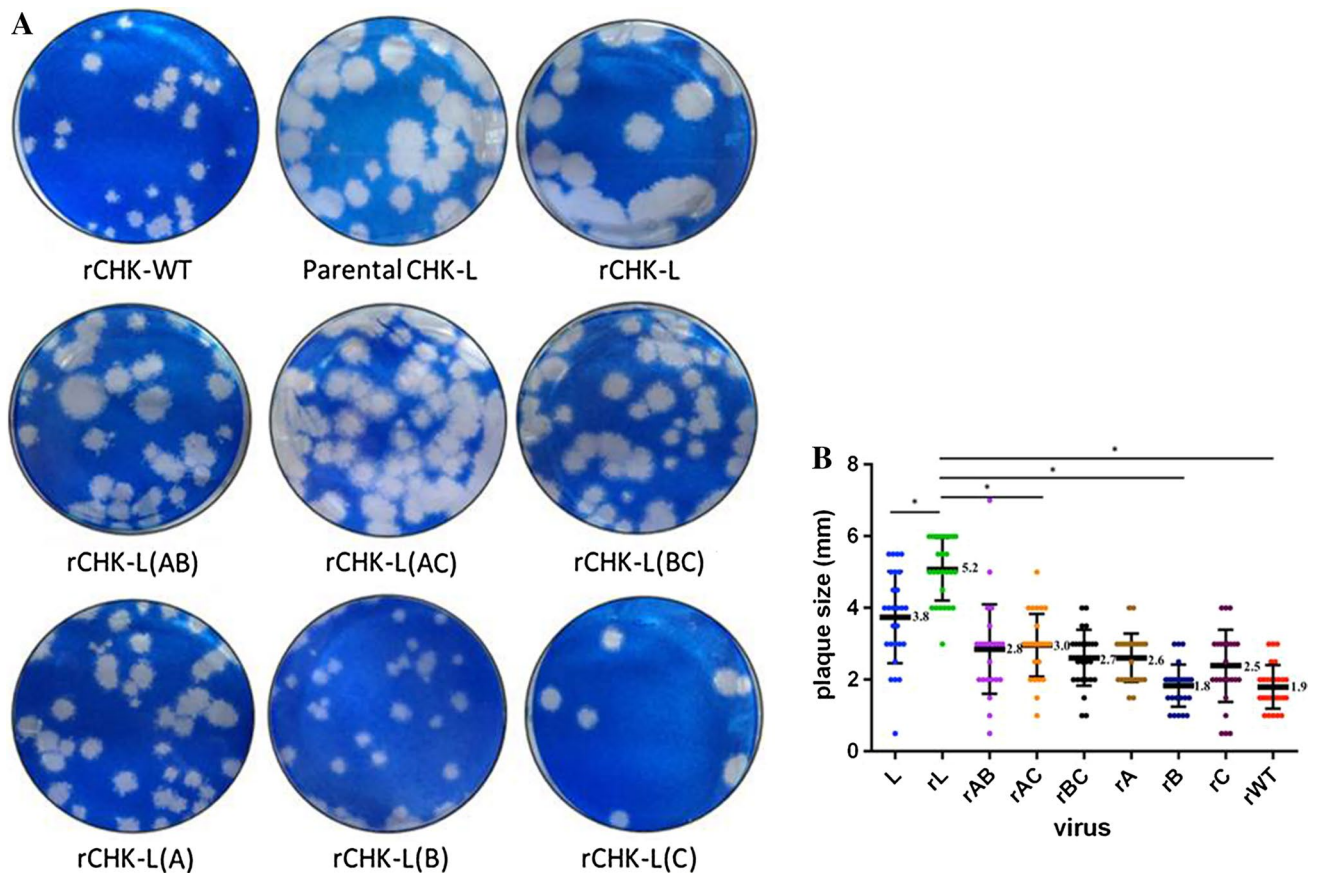
**Fig. 4 a** Plaque morphologies of recombinant viruses compared with the parental CHK-L. rCHK-WT, rCHK-L, rCHK-L(AB), rCHK-L(AC), rCHK-L(BC), rCHK-L(A), rCHK-L(B), rCHK-L(C), and parental CHK-L were subjected to plaque assay in Vero cells. The infected cells were fixed and stained at day 5 post-infection. Each circle represents one well of a 6-well plate. **b** Plaque sizes of recombinant viruses compared with that of parental CHK-L. The parental (L) and recombinant (rL, rAB, rAC, rBC, rA, rB, rC, rWT) CHIKVs were subjected to plaque assay in Vero cells and incubated for 5 days. The diameter of thirty plaques of each virus was measured in millimeters. Each dot represents one plaque; bold black lines are averages and error bars show SD. Asterisks show $p < 0.01$

are required for invasion, replication or transmission of the virus.

Previously, we observed that CHIKV produced from Vero cells showed a larger plaque morphology as compared to its previous passage [29]. In addition to our observation, Lin and colleagues also showed that Vero cell adapted-enterovirus 71 produced larger plaque sizes than the original enterovirus 71 [30]. Therefore, Vero cells were used for the selection of a large plaque variant of CHIKV. Following large plaque selection and propagation in Vero cells for 17 passages, the resultant CHK-L displayed a homogenous large plaque phenotype (Fig. 1i).

Viruses with different plaque morphology often display different degrees of virulence. Since adult mice do not succumb to CHIKV infection, we used a suckling mouse model to investigate the pathogenicity of the parental wild-type virus, WT-CHK025, and the large plaque isolate, CHK-L. Although suckling mice might not be a perfect animal model for CHIKV infection, it has previously been used to study CHIKV neurovirulence for vaccine development [20]. In this study only 15.6% of mice inoculated with CHK-L were alive after 21 days, while 74% of the mice inoculated with WT-CHK025 were alive after the same amount of time, indicating that CHK-L is more pathogenic than WT-CHK025. This result is in agreement with observations for other viruses that viruses with larger plaque sizes are more pathogenic than viruses that produce smaller plaques [19, 20, 25, 31].

The complete genomic sequence of WT-CHK025 was determined. Phylogenetic analysis confirmed that this virus belongs to the ECSA lineage based upon whole genome sequence analysis, and moreover showed that amino acid position 226 of the E1 protein is valine, which is similar to other CHIKV isolates from the recent outbreak in Thailand and neighboring countries [32–35]. Previous studies suggested that this E1:A226V mutation enhances viral infectivity in *A. albopictus* mosquitoes, which led to epidemics in areas that are predominated by *Ae.albopictus* including

the southern part of Thailand where the serum from which the virus was isolated was collected [6, 16].

Comparison of the entire genomic sequences of WT-CHK025 and CHK-L showed 6 nucleic acid differences, 4 of which are located in the coding region. However, confirmation with an additional two clones of large plaque virus validated the presence of only 3 nonsynonymous mutations, which lead to 3 amino acid changes, nsP2 ($P^{618} \rightarrow L$), nsP3 ($G^{117} \rightarrow R$), or E2 ($N^{187} \rightarrow K$). Of all 3 positions, only nsP3 ($G^{117} \rightarrow R$) can be found in one of the other 40 CHIKV primary isolates (data not shown). In addition, using reverse genetics, it was shown that the synergistic effects of all three amino acids were required to acquire the large plaque morphology.

The nonstructural proteins of alphaviruses are expressed as a polyprotein, nsP1234, which undergoes sequential cleavage by the nsP2 protease activity into nsP123+nsP4, nsP1+nsP23+nsP4 and ultimately to individual nsP1, 2, 3, and 4. Each cleavage product is required at a different step of viral replication, and the nsP123 polyprotein is required for negative strand RNA synthesis at the beginning of replication while the cleaved individual nsP1, 2, 3 are subsequently required for positive strand RNA synthesis [13, 36]. Therefore, changing from a polar proline to a nonpolar leucine at position 618, which is located in the protease domain of nsP2, might alter RNA synthesis in a positive way, resulting in a large plaque phenotype. By interacting with several cellular proteins, nsP3 is involved in several aspects of viral replication, including forming the replication complex and counteracting cellular responses [10, 14, 37, 38]. Interestingly, the nsP3 mutation ($G^{117} \rightarrow R$) is located in the macro domain of this protein, which has been shown to be important in Sindbis virus replication and in mediating pathogenicity in mice [39], and therefore changing this amino acid in the protein might alter CHIKV replication and viral pathogenicity. The CHIKV E2 protein functions as the receptor binding protein [10]. A previous study using a chimeric alphavirus showed that the mutations in E2 that increase the positive charge of the protein can enhance the binding of the virus to heparan sulfate, a putative alphavirus receptor, leading to more efficient viral entry and replication in Vero cells [40]. The change from an uncharged asparagine to a positively charged lysine in E2 of CHK-L might have a similar effect resulting in increased viral entry and replication, and consequently resulting in a larger plaque size. In light of the fact that any combination of double mutation resulted in comparable smaller plaque sizes, the three mutations probably work synergistically in the contribution to the large plaque phenotype. However, further study is required to better understand the mechanism responsible for this phenotype.

**Author contributions** Study conception and design: RP. Acquisition of data: BT, TJ, SO, SY. Materials/reagents contributors: SY, SU, RP. Analysis and interpretation of the data: BT, TJ, SU, RP. Drafting of manuscript: RP.

## Compliance with ethical standards

**Conflict of interest** The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors declare that they have no conflict of interest.

**Ethical approval** All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. The approved protocol number: MB-ACUC 2016/006.

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