

## Detection of Persistent Chikungunya Virus RNA but not Infectious Virus in Experimental Vertical Transmission in *Aedes aegypti* from Malaysia

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**Abstract.** Vertical transmission may contribute to the maintenance of arthropod-borne viruses, but its existence in chikungunya virus (CHIKV) is unclear. Experimental vertical transmission of infectious clones of CHIKV in *Aedes aegypti* mosquitoes from Malaysia was investigated. Eggs and adult progeny from the second gonotrophic cycles of infected parental mosquitoes were tested. Using polymerase chain reaction (PCR), 56.3% of pooled eggs and 10% of adult progeny had detectable CHIKV RNA, but no samples had detectable infectious virus by plaque assay. Transfected CHIKV RNA from PCR-positive eggs did not yield infectious virus in BHK-21 cells. Thus, vertical transmission of viable CHIKV was not demonstrated. Noninfectious CHIKV RNA persists in eggs and progeny of infected *Ae. aegypti*, but the mechanism and significance are unknown. There is insufficient evidence to conclude that vertical transmission exists in CHIKV, as positive results reported in previous studies were almost exclusively based only on viral RNA detection.

### INTRODUCTION

Chikungunya virus (CHIKV) is an arthropod-borne alpha-virus belonging to the *Togaviridae* family, which is transmitted to humans mainly by *Aedes aegypti* and *Aedes albopictus*. In Asia, *Ae. aegypti* mosquitoes are the primary vector in an urban cycle of transmission. CHIKV causes acute fever, myalgia, rash, and arthralgia, and the latter may persist for years.<sup>1</sup> There are three main CHIKV genotypes: West African, East/Central/South African (ECSA), and Asian. Recent strains of the ECSA genotype form two sublineages, Indian and Indian Ocean.<sup>2</sup> Over the past 10 years, CHIKV has reemerged to cause worldwide epidemics affecting millions. These include epidemic ECSA strains spreading from East Africa to the islands and land masses adjacent to the Indian Ocean and Asia (Indian sublineage), including Malaysia,<sup>2</sup> and the Asian genotype spreading to the Americas.<sup>1</sup>

Vertical transmission of arboviruses occurs from an adult female mosquito to its eggs and progeny. This may be an important source of maintenance for arboviruses such as dengue virus<sup>3–5</sup> and West Nile virus,<sup>6,7</sup> particularly during unfavorable environmental conditions. Inconsistent findings are reported for the existence of vertical transmission of CHIKV; if it does occur, it is infrequent.<sup>8,9</sup> The aim of this study was to determine the occurrence of experimental vertical transmission using CHIKV from each ECSA sublineage (Indian and Indian Ocean) in *Ae. aegypti* mosquitoes from Malaysia. CHIKV undergoes local adaptation to mosquitoes in different geographical settings, leading to increased midgut replication and dissemination, although effects on vertical transmission have not been specifically studied.<sup>10</sup> If vertical transmission is shown to occur in Malaysian mosquitoes, we examined whether it would occur to a greater extent in CHIKV of the Indian sublineage, which caused large outbreaks in Malaysia, rather than CHIKV of the Indian Ocean sublineage, which have not been reported in Malaysia.

### MATERIALS AND METHODS

**Infectious clones.** Full-length infectious cDNA clones were constructed as previously described<sup>11</sup> for the two CHIKV strains used: ICRES1 (based on LR2006\_OPY1, a virus of Indian Ocean sublineage, isolated in Reunion Island; GenBank accession number: DQ443544) and SGP011 (based on SGEHICH13508, a virus of the Indian sublineage isolated in Singapore; FJ445511),<sup>12</sup> which is closely related to Malaysian viruses (Figure 1).

Viruses were rescued by electroporation of in vitro transcribed infectious transcripts from corresponding cDNA clones in BHK-21 cells (ATCC no., CCL-10). Plasmids were first linearized with *Not I* restriction endonuclease, followed by in vitro transcription from the minimal SP6 promoter as described previously.<sup>13</sup> Approximately 10 µg of in vitro transcribed RNA were electroporated into 10<sup>6</sup> BHK-21 cells using an electroporator with a 4 mm gap electroporation cuvette. Cells were pulsed twice with a square wave protocol, with a 240V pulse for 3 seconds and a time constant after each pulse of 25 milliseconds. Cell culture supernatants were harvested 24 hours postelectroporation and virus titers in *Ae. aegypti* were measured by plaque assay.<sup>13</sup> Mosquitoes were fed with infectious blood meal containing either of the CHIKV strains used, and 10 individual mosquitoes were collected at each time point of 0, 1, 2, 3, 5, 7, and 10 days postinfection (dpi).

**Preparation of homogenate from mosquitoes and eggs.** To determine replication kinetics of infectious clones, midguts were dissected out using clean needles which were soaked in 70% alcohol between each mosquito. To determine the presence of infectious virus in parental and adult progeny mosquitoes, whole mosquitoes were homogenized. Mosquito eggs were homogenized in pools from an individual parent mosquito. All samples were homogenized in tubes containing zirconium beads and 500 µL of serum-free medium.

**Plaque assay.** The virus titers of mosquito and egg homogenates were quantified using plaque assay. Samples were serially diluted, and 400 µL of each dilution were added to duplicate wells of six-well plates containing 80–90% confluent monolayers of BHK-21 cells, supplemented with

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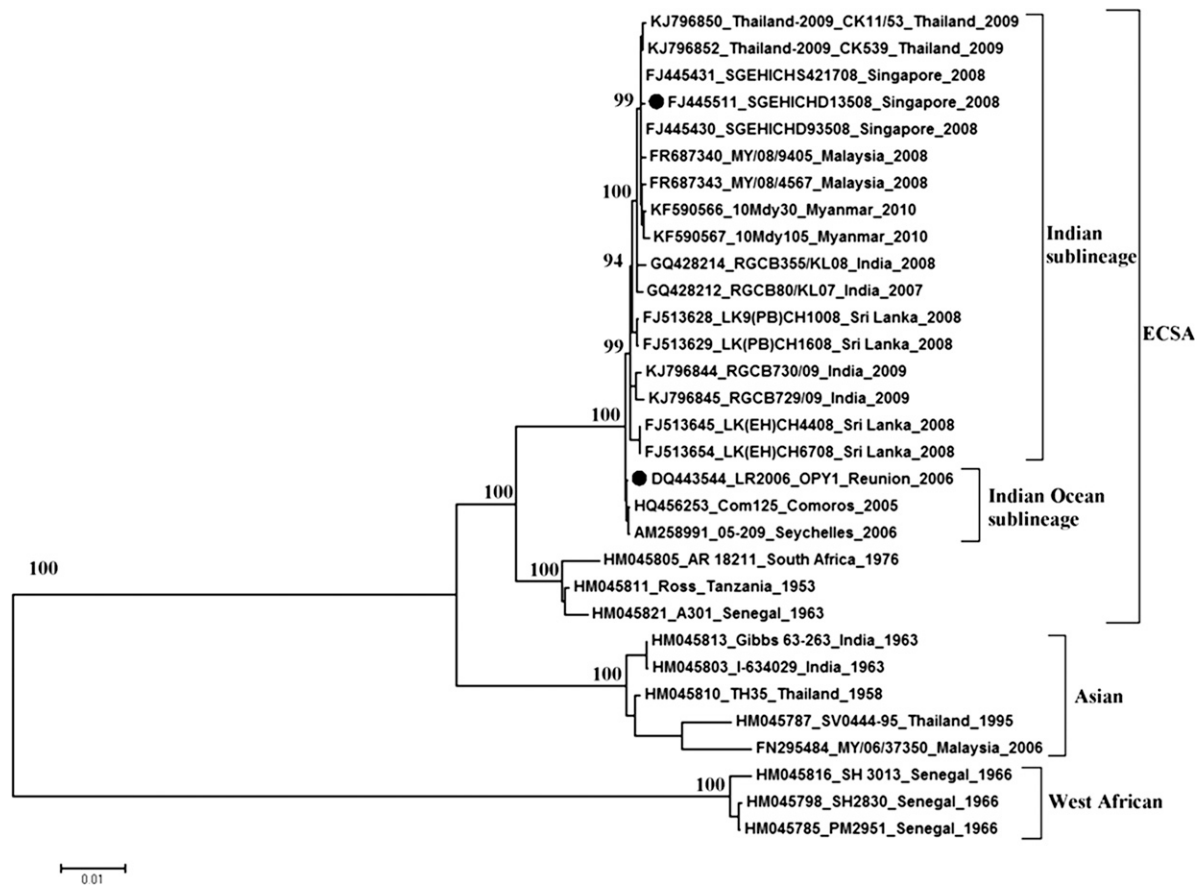


FIGURE 1. Phylogenetic analysis of full coding sequences of chikungunya virus (CHIKV). A maximum likelihood tree was drawn with MEGA5, using the general time reversible model with proportion of invariant sites and gamma-distributed rates among sites (GTR + I + G), and inferred following bootstrap analysis with 1,000 replicates. The scale shows branch lengths measured in the number of substitutions per site. The three main genotypes are shown: West African, Asian, and East/Central/South African (ECSA). The infectious clones ICRES1 and SGP011 used in this study were based on the sequences with accession numbers DQ443544 and FJ445511, respectively, and are indicated by (●).

growth medium containing carboxymethylcellulose sodium salt. Plaque forming units (pfu) were counted after staining with crystal violet. Virus titers are reported as pfu/mL of homogenate.

**Vertical transmission of CHIKV in mosquitoes.** Laboratory-reared *Ae. aegypti* (Seputeh strain, collected in Kuala Lumpur, Malaysia) were used. Female mosquitoes aged 3–6 days were fed with  $6.1 \times 10^5$  pfu/mL of each CHIKV strain in blood donated by Wan Yusof Wan Sulaiman, who is seronegative for CHIKV antibodies.<sup>2</sup> Control mosquitoes were fed uninfected blood. The mosquitoes were provided the blood meal using a Hemotek feeder (Hemotek Ltd., United Kingdom) at 37°C for 1 hour in the dark. Fully engorged mosquitoes were maintained at  $28 \pm 1^\circ\text{C}$  with 88% relative humidity and a 12 hour: 12 hour photoperiod. Mosquitoes were fed with 10% sucrose supplemented with vitamin B complex until 7 dpi, then starved for 24 hours before being fed a clean blood meal. Fully engorged mosquitoes were individually separated and set for oviposition, and eggs from the second gonotrophic cycle were collected at 11–13 dpi. Parental adult homogenates were tested with reverse transcription-polymerase chain reaction (RT-PCR) targeting the region encoding for E1 protein of CHIKV, as well as plaque assay to detect infectious virus. All PCR-positive homogenates prepared from parental adults were confirmed to have infectious virus titers of at least  $10^4$  pfu/mL. Eggs

from PCR-positive and plaque assay-positive parental adults were further processed, with half being homogenized for RT-PCR (regions encoding E1 and the nonstructural proteins nsP1 and nsP3) and plaque assay, while the other half were reared under standard laboratory conditions until adulthood.

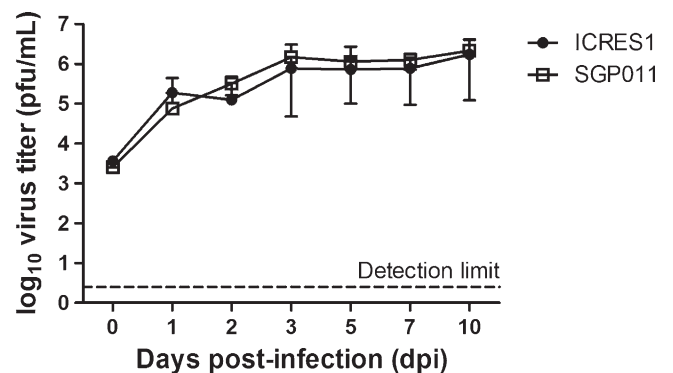


FIGURE 2. Replication kinetics of two different strains of chikungunya virus (CHIKV) infectious clones in the midguts of *Aedes aegypti*. Virus titers were measured by plaque assay in BHK-21 cells and refer to plaque forming units per milliliter of homogenate. Error bars represent standard deviations of means of positive titers from 4 to 10 infected individual mosquitoes at each time point.

TABLE 1

Detection rates of CHIKV RNA and infectious virus in *Aedes aegypti* eggs and progeny

	CHIKV ICRES1 blood-fed parental mosquitoes (N = 11)		CHIKV SGP011 blood-fed parental mosquitoes (N = 21)	
	RNA	Infectious virus	RNA	Infectious virus
Eggs (pools)	5/11 (45.5%)	0	13/21 (61.9%)	0
Progeny adult mosquitoes	4/22 (18.2%)	0	1/28 (3.6%)	0

CHIKV = chikungunya virus.

Progeny adult homogenates were also analyzed by RT-PCR targeting the region encoding E1 and plaque assay. The eggs from the first gonotrophic cycle were not examined as vertical transmission is more likely to occur in second and subsequent cycles following infection with CHIKV,<sup>14</sup> probably due to a longer extrinsic incubation period allowing replication in the ovaries.

**Reverse transcription-polymerase chain reaction.** Previously published RT-PCR protocols were used for the detection of regions encoding E1,<sup>15</sup> nsP1,<sup>15</sup> and nsP3<sup>16</sup> of CHIKV. The identities of E1, nsP1, and nsP3 region-specific products were all confirmed by sequencing.

**Transfection of CHIKV RNA.** To test if CHIKV RNA detected in eggs is infectious, CHIKV RNA was extracted

from the egg homogenates and transfected into BHK-21 cells using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's protocols. Two different positive controls were used, which successfully yielded infectious virus: CHIKV RNA alone, and, to exclude inhibitory effects from mosquito RNA, CHIKV RNA mixed with extracted total RNA from mosquito eggs which were PCR-negative for CHIKV. Cell supernatants were collected at 2 dpi and tested for CHIKV RNA and infectious virus.

## RESULTS AND DISCUSSION

Both infectious clones replicated stably and similarly in the midguts of *Ae. aegypti* (Figure 2). Both ICRES1 and SGP011 reached similar peak titers of  $1.7 \times 10^6$  pfu/mL and  $2.1 \times 10^6$  pfu/mL, respectively, at 10 dpi.

A total of 32 adult females that laid eggs were confirmed to be positive by RT-PCR. Of these, 11 were infected with ICRES1 and 21 were infected with SGP011 (Table 1). All control mosquitoes tested negative by RT-PCR. CHIKV RNA was detected from 18/32 (56.3%) of the total pools of eggs from the second gonotrophic cycle, and 5/50 (10%) of the adult mosquitoes reared from these eggs. There were no significant differences in RNA detection rates between the two virus strains used. However, although CHIKV RNA was detectable in the eggs and progeny of infected

TABLE 2

Summary of previous studies of vertical transmission of chikungunya virus (CHIKV) in *Aedes* mosquitoes

Location	Species	Mosquitoes tested	Assays used			Reference
			RT-PCR	IF	Virus culture	
India	<i>Aedes aegypti</i>	Larvae and adult progeny of experimentally infected mosquitoes	<i>Ae. aegypti</i> : 8/30 larvae (26.6%) and 14/33 adults (42.4%)	Not done	Not done	14
India	<i>Ae. aegypti</i> , <i>Ae. albopictus</i>	Adult progeny of experimentally infected mosquitoes (three gonotrophic cycles)	Not done	<i>Ae. aegypti</i> : 0/3,814 (0%); <i>Ae. albopictus</i> : 0/1,754 (0%)	Not done	17
Thailand	<i>Ae. aegypti</i> , <i>Ae. albopictus</i>	Male adults collected from affected sites	<i>Ae. aegypti</i> : 6/28 pools (21.4%); <i>Ae. albopictus</i> : 2/2 pools (100%)	Not done	Not done	18
South Africa	<i>Ae. aegypti</i> , <i>Ae. fuscifer</i>	Adult progeny of experimentally infected mosquitoes	Not done	Not done	In mice, <i>Ae. aegypti</i> : 0/3,158 adults (0%); <i>Ae. fuscifer</i> : 0/1,395 adults (0%)	19
India	<i>Ae. albopictus</i>	Adults reared from larvae from affected sites	<i>Ae. albopictus</i> : 3/3 pools (100%)	Not done	Virus detected, after three passages in Vero cells	20
Reunion Island	<i>Ae. albopictus</i>	Adults reared from larvae from affected sites	<i>Ae. albopictus</i> : 2/502 adults (0.4%)	Not done	Not done	21
Reunion Island	<i>Ae. albopictus</i>	Adult progeny of experimentally infected mosquitoes (G1 and G2)	G1: 0/1,675 (0%); G2: 0/1,709 (0%)	Not done	Not done	22
Italy	<i>Ae. albopictus</i>	Adult progeny of experimentally infected mosquitoes (three gonotrophic cycles)	<i>Ae. albopictus</i> : 3/689 adults (0.4%)	Not done	Not done	8
Madagascar	<i>Ae. albopictus</i>	Adults reared from larvae from affected sites	<i>Ae. albopictus</i> : 5/23 pools (21.8%)	Not done	No growth in AP61 cells	9

CHIKV = chikungunya virus; G1 = first gonotrophic cycle; G2 = second gonotrophic cycle; IF = immunofluorescence assay; RT-PCR = reverse transcription-polymerase chain reaction.

*Ae. aegypti*, none was found to contain infectious virus detectable by plaque assay. The homogenate prepared from progeny adults were further passaged another three times in BHK-21 cells, after which neither infectious virus nor RNA was detectable. CHIKV RNA extracted from the eggs homogenate was also transfected into BHK-21 cells, to see if infectious virus could be generated. The transfected CHIKV RNA from the eggs homogenate produced no infectious virus, and CHIKV RNA was no longer detectable in the transfected BHK-21 after two further passages. This suggests that the CHIKV RNA detected in the eggs homogenate was noninfectious.

For comparison, previous studies of vertical transmission of CHIKV in *Aedes* mosquitoes were summarized (Table 2). RT-PCR was the most common method used to detect CHIKV, in seven of nine studies,<sup>8,9,14,18,20–22</sup> and only one of those failed to detect CHIKV RNA.<sup>22</sup> RNA detection rates otherwise ranged between 0.4% and 100%, in both experimentally and naturally infected samples. Notably, only five studies used methods to detect either viral protein expression (immunofluorescence staining<sup>17,22</sup>) or viable infectious virus (cell culture<sup>9,20</sup> and in vivo inoculation<sup>19</sup>). Only one of these studies demonstrated the presence of infectious virus, in *Ae. albopictus* adults reared from larvae from outbreak sites; this required three passages of obtained materials in Vero cells, and ultimately yielded unusually high levels of  $10^{10-11}$  pfu/mL in cell culture supernatants.<sup>20</sup> Thus, the inconsistent results reported for vertical transmission in CHIKV appear to be mainly due to the assay used to detect the virus: viral RNA has been detected by many researchers, while infectious virus has not.

Definitive demonstration of successful vertical transmission of infectious CHIKV is important, as this is a significant gap in the understanding of CHIKV maintenance outside the human host. Other alphaviruses have shown evidence of viable vertical transmission in mosquitoes and other insects. Examples of these include: western equine encephalitis virus, isolated from adult *Aedes dorsalis* collected as larvae<sup>23</sup>; Ndumu virus, cultured from *Culex pipiens* grown from field-collected larvae<sup>24</sup>; and Buggy Creek virus, isolated from eggs of *Oeciacus vicarius* swallow bugs.<sup>25</sup>

On the basis of the findings of this and other studies, it should be concluded that detection of viral RNA itself is insufficient to confirm the occurrence of vertical transmission of CHIKV. It is possible that the detection of only viral RNA may be due to the relative insensitivity of the plaque assay technique used, which had a limit of detection of 2.5 pfu/mL, while the limit of detection of the RT-PCR assay used to detect the region encoding E1 was as low as 0.08 pg of CHIKV RNA. Alternatively, viral RNA may represent inactivated virus, which may remain detectable even in dead mosquitoes stored for many weeks at 28°C.<sup>26</sup> The desiccation-resistant nature of *Aedes* eggs may enhance the stability of viral nucleic acid. Viral RNA may also represent a defective or latent form of persistent virus. In our study, the failure to produce infectious virus following transfection of CHIKV RNA obtained from the egg homogenates may be because the detected CHIKV RNA is incomplete, defective, or possibly in the form of a double-stranded replication intermediate, all of which would not allow translation of the nonstructural proteins required to start the infection cycle. Long-term persistence of the flavivirus West Nile virus RNA, but not live

virus, has been reported in avian hosts up to 36 weeks postinfection.<sup>27</sup> However, it is not known if the virus can recrudescence to render the bird infectious to arthropods once more. Similarly, bluetongue virus RNA but not infectious virus was detected in *Culicoides* midge larvae from the field.<sup>28</sup> Segments of the viral RNA encoding the outer capsid genes, required for growth in insect cells, were downregulated; it was hypothesized that this allows bluetongue virus to survive as a persistent, noncytotoxic infection of the insect host which is inactive over winter, but with the ability to reactivate later. Another alphavirus, Sindbis virus, has been shown to persist in a nonproductive RNA form in immunodeficient mouse brains, and can be reactivated as infectious virus after several months.<sup>29</sup> It has been recently reported that Sindbis virus may replicate as noncapped genomes with extremely reduced yield of infectious virus.<sup>30</sup> Therefore, the possibility exists that CHIKV RNA is present in a latent form in eggs or progeny before an unknown reactivating stimulus. In general, our findings are analogous with other studies which have been unable to isolate infectious virus from mammals with persistently detectable CHIKV RNA.<sup>31,32</sup>

In conclusion, CHIKV RNA persists for prolonged periods in mosquito eggs and is also detectable in hatched adults. However, in the absence of detection of infectious virus particles, this is not definitive evidence of vertical transmission, which remains largely unproven for CHIKV. Future research should explore the potential significance of persistent viral RNA in eggs and progeny, and standardize the detection assay to confirm vertical transmission.

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