

Short Report: Persistence of Viral RNA in Chikungunya Virus-Infected *Aedes aegypti* (Diptera: Culicidae) Mosquitoes after Prolonged Storage at 28°C

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Abstract. Experiments were conducted to determine the persistence of chikungunya viral (CHIKV) RNA in experimentally infected *Aedes aegypti* mosquitoes stored for prolonged periods at 28°C. Intra-thoracically inoculated mosquitoes with confirmed positivity were killed by quick freezing at –80°C, applied to sticky tape, and stored at 28°C with 80 ± 5% relative humidity (RH). At weekly intervals, five mosquitoes were removed from the tape randomly and assayed individually for detection of viral RNA by reverse transcriptase-polymerase chain reaction (RT-PCR). CHIKV RNA was detected up to 12 weeks in dry mosquitoes by RT-PCR. Virus could not be isolated either in cell culture or in the suckling Swiss-albino mouse system at any stage. This study demonstrated the persistence of CHIKV viral RNA up to 12 weeks when stored at 28°C with RH 80 ± 5%. This finding will have significance in CHIKV surveillance programs in mosquito populations or field-based studies in countries where maintenance of a cold chain is a concern.

Chikungunya fever is an arthropod-borne disease caused by chikungunya virus (CHIKV) belonging to genus *Alphavirus*, family *Togaviridae*. CHIKV has re-emerged in India, Indian Ocean Islands, and South East Asia in an explosive outbreak affecting ~6 million people in 2005–06.¹ During the resurgence, the virus caused severe morbidity and clinical complications that were hitherto unknown of CHIKV.² Neurological, ophthalmological, renal, and cutaneous involvement was more evident during the recent outbreak in addition to encephalitis/encephalopathy.^{1–3} The CHIKV-associated mortality was very high in La Reunion Island and India and accounted for several thousand deaths.⁴ The virus is transmitted to humans by the bite of infected *Aedes aegypti* mosquitoes, the principal vector of chikungunya in urban areas and *Ae. albopictus* mosquitoes in peri-urban areas.² The recent mutation in the CHIKV genome has helped the virus to adapt to the new vector, *Aedes albopictus* for rapid dissemination and transmission.⁵ Vector control is the only means to break virus transmission as no effective therapies or vaccines are in place for the virus. Proactive surveillance of vector species for detection of CHIKV is an accepted mode of surveillance where wild-caught mosquitoes are screened routinely as part of the virus detection program. However, a major concern was the maintenance of a cold chain during transport of mosquitoes from field to laboratory in many tropical countries. The recent report of dengue virus detection in dried *Ae. aegypti* mosquitoes by reverse transcriptase-polymerase chain reaction (RT-PCR)⁶ have prompted us to take up a study in the same direction. In this communication, we report the methodology of mosquito storage and the persistence of CHIKV RNA in experimentally infected, dried, and stored *Ae. aegypti* mosquitoes using RT-PCR.

In this study, laboratory-reared 2- to 3-day-old adult female *Ae. aegypti* mosquitoes ($N = 150$) were inoculated intra-thoracically with ~0.2 µL of CHIKV (strain no. 061573) suspension as described earlier.⁷ In brief, mosquitoes were immobilized by keeping them over wet ice for 5–10 min and

inoculated with virus suspension in the membranous area of the mesothorax between the spiracle and sternoplural region under a dissecting binocular microscope. Inoculation was carried out using a calibrated capillary needle and syringe plunger and the whole procedure was conducted inside a mosquito proof enclosure. After inoculation, the mosquitoes were placed in plastic mosquito holding jars, provided a diet of 10% glucose (cotton swab soaked in glucose solution), and incubated at 28°C with 80 ± 5% relative humidity (RH). On Day 6 post-infection (PI), 10 mosquitoes were removed randomly and tested for the presence of CHIKV antigen in the head by immunofluorescence assay (IFA) as described by Dhanda and Ilkal.⁸ After confirmation of infectivity in all the tested specimens, the rest of the females were frozen at –80°C for 30 min, removed from the freezer, stuck to sticky adhesive tape (Johnson and Johnson, Mumbai, India), and stored at 28°C with 80 ± 5% RH. Female mosquitoes of the same age group were inoculated with the diluent (0.75% bovine albumin in phosphate buffered saline (BAPS)), served as controls, and were treated similarly as the test mosquitoes. At weekly intervals, from Week 1 to Week 14, five infected and five control mosquitoes were removed randomly from the sticky adhesive tapes and assayed individually to detect CHIKV RNA by RT-PCR. In brief, individual dry mosquitoes were triturated in 250 µL of 0.75% BAPS (pH 7.2) using chilled mortar and pestle. The suspension was collected in an ependorf tube, centrifuged at 10,000 rpm for 1 hr, and the supernatant was collected. It was Millipore filtered (0.22 µm) and 140 µL filtrate was used for RNA extraction using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RT-PCR was carried out targeting partial sequences of NS4 and E1 genes as described earlier.⁹ Cycling conditions were 1 cycle at 94°C for 5 min; 35 cycles each of 94°C (1 min), 50°C (1 min), and 68°C (1.5 min); followed by final extension of 7 min at 68°C. The products were visualized on 1.2% agarose gel using a gel documentation system (Alpha Innotech).

All 10 inoculated mosquitoes harvested on Day 6 PI were found to be positive for CHIKV antigen by IFA using CHIKV antiserum raised in mice. The presence of CHIKV antigen in all 10 randomly selected mosquitoes made us assume 100% positivity in the inoculated mosquitoes. The RT-PCR followed by gel electrophoresis showed CHIKV RNA positivity up to

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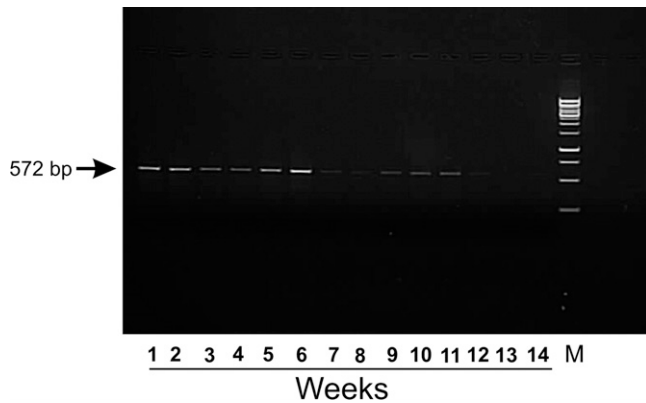


FIGURE 1. Gel electrophoresis of reverse transcriptase-polymerase chain reaction (RT-PCR) amplicon of chikungunya viral (CHIKV) RNA extracted from dried mosquitoes stored at $28 \pm 2^\circ\text{C}$, from Week 1 to Week 14. M = 250 bp ladder; Lane 1–14 CHIKV samples.

12 weeks (Figure 1). The band intensity of samples up to Week 11 was high, whereas a faint band appeared for the Week 12 sample. No band was obtained for Week 13 and Week 14 samples, and this could be caused by either complete degradation of RNA or the amount of RNA below the limit of detection. The study has clearly demonstrated CHIKV RNA persistence up to a maximum of 12 weeks under the above conditions in mosquitoes. All the mock-infected samples tested negative (data not shown). Attempts to isolate virus from the mosquito suspensions using suckling mice (Swiss albino) and tissue culture were futile despite inoculating the suspensions for two consecutive passages.

The sticky tape method for transport of mosquitoes from CHIKV-endemic areas for CHIKV RNA detection in the laboratory using RT-PCR would play an important role in the surveillance of CHIKV in field-collected mosquitoes. The technique has been successfully applied in several studies to screen mosquitoes for arboviruses.⁶ Bangs and coworkers¹⁰ demonstrated the persistence of dengue viral RNA in dried mosquitoes up to 30 days after application to the lure. Subsequent studies by the authors demonstrated the persistence of dengue virus RNA in dried *Ae. aegypti* mosquitoes for 13 weeks under tropical conditions.⁶ The persistence of Western equine encephalomyelitis and St. Louis encephalitis viral RNAs in mosquitoes up to 20 days in the absence of a cold chain has also been reported.¹¹ Similar results were also reported for West Nile and Japanese encephalitis viruses stored at room temperature for 14 days.^{12,13} Guzman and colleagues¹⁴ demonstrated the persistence of Venezuelan equine encephalitis virus RNA for 40 days. This study has demonstrated the persistence of CHIKV RNA up to 12 weeks in mosquitoes stored at 28°C with high humidity ($80 \pm 5\%$).

The RT-PCR has been a reliable tool for specific and rapid detection of CHIK viral RNA in serum and mosquito samples.^{9,15} The technique has been used successfully for rapid diagnosis of many viral agents during outbreaks.³ The applicability of the technique in this study to detect CHIKV RNA in dry mosquitoes up to 12 weeks shows its efficacy. The persistence of CHIKV RNA at 28°C with 80 ± 5 RH% up to 12 weeks in dry mosquitoes on sticky traps is a significant observation and has considerable epidemiological importance. Though CHIKV is comparatively stable at room temperature in serum samples,¹⁶ it is not clear how the viral RNA is pre-

served in dead mosquitoes for prolonged periods. However, this study clearly shows that maintaining of cold chain can be omitted when RT-PCR is used as the diagnostic technique for RNA detection. This technique will most appropriately fit during field-based virus surveillance in mosquitoes. The technique will have significant application, especially in third world countries where limited resources are available to carry out virus surveillance in mosquitoes and other virus vectors.

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