

Rapid detection of chikungunya virus in laboratory infected *Aedes aegypti* by Reverse-Transcriptase-Polymerase Chain Reaction (RT-PCR)

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Abstract. A study of chikungunya virus was carried out to establish Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) as a rapid detection technique of the virus. The susceptibility of lab-colonized *Aedes aegypti* to chikungunya virus was also determined. Artificial membrane feeding technique was used to orally feed the mosquitoes with a human isolate of chikungunya virus. A total of 100 fully engorged female *Ae. aegypti* were obtained and maintained for 7 days. Seventy of them survived and then pooled at 10 individuals per pool. Total RNA was extracted from the samples and RT-PCR amplifications were carried out. Five out of 7 pools showed positive PCR band at 350-bp, indicating *Ae. aegypti* is a potential vector of chikungunya virus. The minimum infection rate (MIR) was 71% within these laboratory colonies. RT-PCR is a sensitive technique that is useful in detecting infected mosquitoes in epidemic areas. This technique can be used as a rapid detection method and provide an early virologic surveillance systems of chikungunya virus infected mosquitoes.

INTRODUCTION

Chikungunya virus, a mosquito-borne togavirus belonging to the genus Alphavirus has caused numerous well-documented outbreaks and epidemics in Southeast Asian and African countries. Sporadic cases have also been reported in India and Burma (Thaung, 1975; Thaikruea *et al.*, 1997; Laras *et al.*, 2005). In Malaysia, chikungunya cases were never reported until 1998-1999 during which an outbreak of chikungunya virus occurred in Klang, Malaysia, between December 1998 and February 1999 (Lam *et al.*, 2001). Although there was serological evidence of its presence in Malaysia, chikungunya virus has not been known to be associated with clinical illness in human in the country.

Chikungunya fever is characterized by sudden onset of chills and fever, headache, nausea, vomiting, arthralgia and rash. Compared with dengue, chikungunya is

distinguished by a briefer incubation period and febrile episode, by persistent arthralgia in some cases, and by the absence of fatalities. Because the clinical symptoms of chikungunya virus infection often mimic those of dengue fever and chikungunya virus co-circulates in regions where dengue virus is endemic, it has been postulated that many cases of dengue virus infection are misdiagnosed and that the incidence of chikungunya virus infection is much higher than reported (Powers *et al.*, 2000).

Chikungunya virus is enzootic in many countries in Asia and throughout tropical Africa. Various species of *Aedes* mosquitoes have been incriminated as vectors or potential vectors of chikungunya. In Asia the virus is transmitted from primates to humans almost exclusively by *Aedes aegypti*, while various aedine mosquito species are responsible for human infections in Africa

(Pfeffer *et al.*, 2002). In Thailand, *Ae. aegypti* and *Ae. albopictus* have been associated with chikungunya outbreaks in 1995 (Thaikruea *et al.*, 1997). *Ae. furcifer* and *Ae. cordellieri* are considered to be epidemic-epizootic vectors during epidemics in South Africa (Diallo *et al.*, 1999), while in West and Central Africa, *Ae. africanus* is the dominant vector (Powers *et al.*, 2000). Chikungunya virus is strictly tropical in distribution, which is clear from its geographical distribution pattern in southern Africa, where the virus is absent from the temperate areas (Jupp & McIntosh, 1985). Large epidemics occur in urban and semi-urban settings where the virus is transmitted by *Ae. aegypti*. The man-biting habits of domestic *Ae. aegypti* appear to vary in countries where chikungunya epidemics have occurred.

Though chikungunya virus diagnosis based on virus isolation is very sensitive, yet it requires at least a week in conjunction with virus identification using monovalent sera. Reverse transcriptase-polymerase chain reaction (RT-PCR) is the most sensitive technique for mRNA detection and quantification currently available. Our Unit has an ongoing programme of transmission experiments to investigate the chikungunya virus vectorial capacity of selected mosquito species. In this study, we have attempted to confirm the possible development of chikungunya virus in laboratory-reared *Ae. aegypti*. RT-PCR will be applied in the detection of chikungunya viral RNA in artificially infected *Aedes aegypti* mosquitoes and based on the available published sequences, the expected 350 base pair was amplified by PCR.

MATERIALS AND METHODS

Mosquitoes

The mosquitoes employed for the experiments were from a laboratory colony maintained in this Institute for more than 30 years. The mosquitoes were maintained at 70-80% relative humidity and 24-25°C.

Chikungunya virus

Chikungunya virus employed in the experiment was originally obtained from the Division of Virology, Nagasaki University, Japan, and had been passaged in *Ae. albopictus* C6/36 cell line. The virus was maintained in the cell culture and incubated at 28°C in Eagle's medium minimum essential medium supplemented with 2% heat-activated fetal calf serum and 0.2mM non essential amino acids. The infected culture fluid was harvested 4-5 days after inoculation and centrifuged. The supernatant was then filtered with 0.22µm filter unit (Nunc) and the virus was concentrated by Integrated Speed Vac system (Savant ISS 100SC) at 14 000rpm for 3 hours.

Artificial membrane feeding and transmission procedure

The artificial membrane feeding technique employed was modified from Graves (1980). Two hundred 4-7 days old female *Ae. aegypti* adults were collected and starved overnight prior to blood feeding. Approximately 30 female mosquitoes were placed into each paper cup.

A glass feeder with water jacket was covered at the bottom by wrapping a small piece of membrane, which was moistened with normal saline. Fresh normal human blood was obtained on the day of blood feeding by using venipuncture and immediately transferred into separate heparinized tubes after which the blood was placed into the feeder. Mosquitoes were membrane-fed on a suspension containing 1ml human blood mixed with 100µl of chikungunya virus in C6/36 cell line to obtain infected samples. Uninfected samples were obtained by feeding the mosquitoes with a suspension containing 1.0ml human blood and 100µl of normal saline.

The blood was presented to the mosquitoes by placing the cups containing mosquitoes below the feeder, with the surface of the nylon netting of the cup in contact with the membrane of the feeder. Water from the water bath at 37°C was allowed to flow through the inlet and

outlet of the artificial feeding system to keep the blood warm. The membrane and blood were replaced each time after feeding to prevent gradual settling of the red blood cells. Each cup of mosquitoes was allowed to feed for approximately 30 minutes to 1 hour.

After feeding, all the mosquitoes in each cup were transferred into a cage. Only fully engorged mosquitoes were collected and reared in the cage accordingly. The mosquitoes from each treatment were maintained for 7 days, fed on 10% sucrose solution enriched with 1% vitamin B complex. After 7 days, the mosquitoes were transferred into sterile eppendorf tubes and kept inside a -20°C freezer for further use. All infectious studies were conducted in an isolated infection room.

Detection of chikungunya virus using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Extraction of RNA

The mosquito pools were homogenized in a sterile homogenizer and RNase were extracted using QIAamp Viral RNA Mini Kit (Qiagen). One hundred and forty microliters homogenate were required from each pool to examine the samples. For positive control, equal volume of cultured cells infected with chikungunya virus was used and for negative control, uninfected cultured cells were used. The extracted RNAs were then kept at -20°C until used.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The method for RT-PCR and electrophoresis was employed from Hasabe *et al.* (2002). Master mix was prepared using Titan One Tube RT-PCR Kit. Each reaction required 9.75ml of double distilled water, 2 μl of dNTP mix, 1.25 μl of DTT, 0.5 μl RNase inhibitor, 5.0 μl of RT-PCR buffer, 0.5 μl of enzyme mix, 0.5 μl of CHIKnsP1-S sense primer (5'- TAG-AGC-AGG-AAA-TTG-ATC-CC-3) and 0.5 μl of

CHIKnsP1-C anti sense primer (5'- CTT-TAA-TCG-CCT-GGT-GGT-AT-3'), to reach 20 μl total volume of master mix.

RNA products were prepared by heating the tubes at 65°C for 5 minutes by block heater. Five microliters of each RNA product was added to the master mix and then centrifuged at 8000 rpm.

The RT step was carried out at 37°C for one hour to produce cDNA which were then amplified by the following PCR steps: 94°C for 3 minutes as initial denaturation, 94°C for 30 seconds as denaturation steps, 54°C for 90 seconds as annealing step and 72°C for 2 minutes as extension step. The cycle was repeated 35 times before final extension at 72°C for 5 minutes. For every RT-PCR run, a positive control (a confirmed chikungunya isolate) and negative control were included.

The PCR products were analysed by performing electrophoresis in a 2.0% Nusieve PCR gel (FC Bio, USA) stained with ethidium bromide and run at about 100 volts. The gel was viewed under ultra violet illuminator (Ultra Lum Ins, Colifornia USA) and the resulting bands were captured with a polaroid camera.

Minimum Infection Rate (MIR)

The proportion of athropods feeding which became infected was determined for each pool of mosquitoes. The MIR was calculated as the number of positive pools \div total number of mosquito tested X 1000.

RESULTS AND DISCUSSION

A total of 100 fully engorged female *Ae. aegypti* were obtained and maintained for 7 days. Seventy of them survived and then pooled for 10 individuals per pool.

The minimum infection rate (MIR) was 71% within these laboratory colonies. The value is considerably higher than those were obtained from the field-caught *Aedes* species reported by Diallo *et al.* (1999) which was 4.0-8.8%. However, from another laboratory infection study by Tesh

et al. (1976), different strains of *Ae. albopictus* showed higher infection rate, which varied between 1.9 to 9.7%.

Figure 1 shows agarose gel electrophoresis of RT-PCR of chikungunya virus. The correct size of DNA product (350-bp) was obtained for each of the positive pools after amplification with chikungunya primers. The PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis. The electrophoresis showed positive RT-PCR result in five out of seven sample pools, indicating the presence of chikungunya virus within these mosquitoes. However, a weak PCR band was observed in one of the positive pools. The strong PCR bands were seen in lane number 2,3,7 and 8, whereas lane number 6 shows a weak band at 350bp.

This study has shown that the expected 350-bp cDNA fragment was amplified from the positive samples investigated. It was similar to the study by Hasebe *et al.* (2002), which effectively achieved 354-bp from the nsP1 genes.

Although only fully engorged mosquitoes were selected after a blood feed, not all become infected. It is probably due to the following viz. not all the mosquitoes pick up the virus during feeding, low infection within the mosquitoes, the virus could not replicate in the mosquitoes or more infected mosquitoes were pooled in positive pools. Presumably the titer of virus in a mosquito would effect its ability to transmit the virus. In general, the susceptibility of a mosquito strain to oral infection with chikungunya virus and the mean virus titer

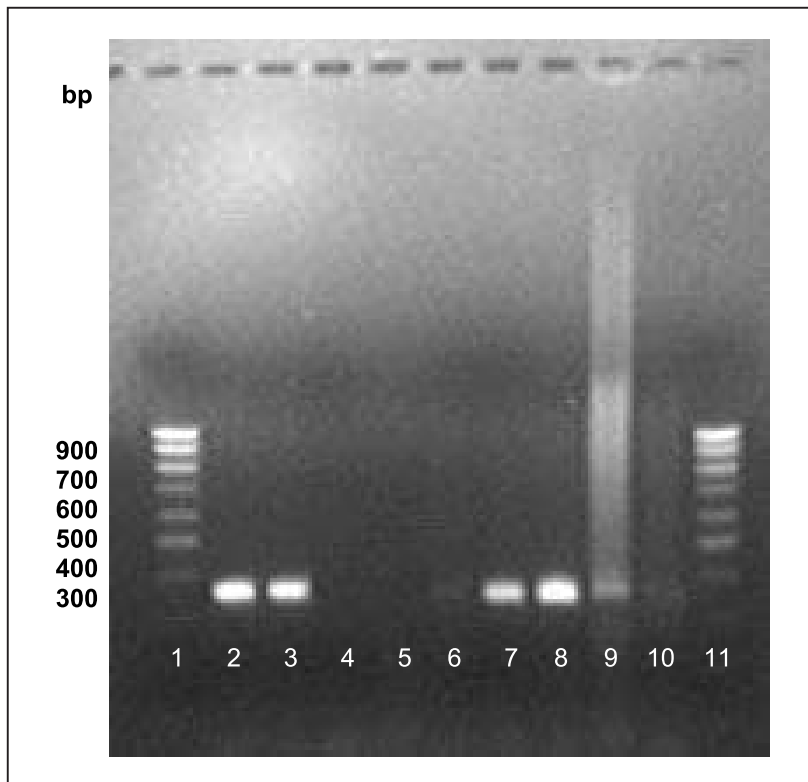


Figure 1. Detection of chikungunya virus in pools of *Aedes aegypti* mosquitoes and electrophoresis of RT-PCR products on 2% agarose gels. Lane 1 and 11 – marker; Lanes 2,3,6,7,8 – positive control of laboratory infected *Ae. aegypti*; lane 4 and 5 – uninfected *Ae. aegypti*; Lane 9 – positive control (C6/36 chikungunya infected cell culture) and Lane 10 – negative control (C6/36 uninfected cell culture).

of infected mosquitoes of that strain were directly related. A higher percentage of mosquitoes could be infected orally with chikungunya virus by increasing the virus dosage. Similar observation was reported by Tesh *et al.* (1976) that there was individual variation among mosquitoes of a given strain not only in susceptibility to oral infection but also in ability to replicate virus.

Lane 6 demonstrated a weak PCR band, which is probably due to the low number of infected mosquito(es) in the pool. Hence, the PCR presumably will demonstrate stronger band under these circumstances: greater number of infected mosquitoes in respective pools, high dosage of viral infection and a favorable vector. For the later study, by Turell *et al.* (1992) found that *Ae. albopictus* appeared to be a more competent laboratory vector of chikungunya virus than *Ae. aegypti*.

It is well known that different mosquito species vary in their susceptibility to experimental infection and in their ability to transmit arbovirus. Studies also have shown that *Aedes* mosquito strains originating from different geographic localities vary not only in their oral susceptibility to infection with chikungunya virus but also in their ability to replicate the virus once infected (Diallo *et al.*, 1999).

Until now, vector of chikungunya in Malaysia is still unknown. *Ae. aegypti* from Malaysia can now be considered a potential vector of chikungunya virus as their vector competence has been proven experimentally in this study. While our work dealt with laboratory colony, the natural chikungunya virus mosquito vectors are yet to be determined.

The chikungunya outbreak in 1998 in Peninsular Malaysia confirmed earlier studies by Marchette *et al.* (1980) which suggested that although human infection with chikungunya virus appears to be of low activity, it is widespread and the virus remains a potential menace and may be responsible for future epidemics (Matusop & Singh, 2000). Furthermore, Malaysia is heavily dependent on migrant workers

from countries where chikungunya virus is endemic. It is speculated that the virus has been re-introduced into the country through the movement of these workers (Lam *et al.*, 2001).

Although chikungunya infection is not fatal, the disease may become a public health threat in the country if the virus is introduced due to the prevalence of *Aedes* mosquito vectors. Early steps should be taken in order to prevent future outbreaks, especially in tropical areas, where vectors are available. RT-PCR can be useful as an early detection warning system to detect infected mosquitoes in epidemic areas. Further characterization of mosquito in the endemic areas and their vector competence for chikungunya virus could also provide valuable information regarding the potential emergence of the viruses in human population.

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