REFRACTORINESS OF *AEDES AEGYPTI* (LINNAEUS) TO DUAL INFECTION WITH DENGUE AND CHIKUNGUNYA VIRUS

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Abstract. In this study, artificial membrane feeding technique was used to orally feed *Aedes aegypti* with dengue and chikungunya viruses. Virus detection was carried out by reverse transcriptase polymerase chain reaction. The study did not detect dual infection of *Ae. aegypti* with dengue and chikungunya virus from the same pool or from individual mosquitoes. Oral receptivity of *Ae. aegypti* to chikungunya virus was higher than that of dengue virus.

INTRODUCTION

Dengue and chikungunya virus are members of the arbovirus that causes disease in primate and humans. There are strong circumstantial evidences that dengue and chikungunya viruses are highly and widely distributed throughout Southeast Asia (Halstead *et al,* 1969; Lam *et al,* 2001). There is also evidence that dengue and chikungunya infections are widespread both in urban and rural population in Myanmar (Thaung *et al,* 1975). Compared with dengue, chikungunya infection is distinguished by a shorter incubation period and febrile episode, persistent arthralgia in some cases and an absence of fatality (Sam, 2006). Because the clinical symptoms of chikungunya virus infection often mimic those of dengue and the 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.

A dual infection of chikungunya and dengue virus in humans was first reported by Myers and Carey (1967). Blood sample from a patient in the acute phase of a dengue 2-like illness presented dual infection with dengue and chikungunya virus. A large outbreak of hemorrhagic fever due to dengue and chikungunya viruses has been reported in Myanmar (Thaung *et al,* 1975). This mixed outbreak has been reported in Andhra Pradesh state, India (December 2005 and February 2006) and in part of Toamasina, Madagascar (January - Febuary 2006) (http://www.who.int.csr/don/2006).

Dual infection with chikungunya and dengue viruses in the mosquito vector in the field has been reported in India (Mourya *et al,* 2001). *Aedes aegypti* and *Ae. albopictus* are vectors of chikungunya and dengue viruses (Mourya *et al,* 2001; Rohani *et al,* 2005). No marked difference in distribution of dengue

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infection in urban and rural areas has been observed but chikungunya seems to be more prevalent in the rural area (Mourya and Yadav, 2006).

As the clinical symptoms of both dengue and chikungunya fever are similar, laboratory confirmation is important, especially in areas where dengue is present. Co-infection of dengue and chikungunya viruses can possibly change clinical spectrum of the disease and consequently, specific treatment may also be affected. Investigations of epidemiological, immunological and entomological aspects of the dual infection need pursuing. Therefore, the aim of this study was to determine possible dual infection of dengue and chikungunya viruses in laboratory-bred *Ae. aegypti.*

MATERIALS AND METHODS

Mosquitoes

Ae. aegypti mosquitoes were from a laboratory colony maintained in the Institute For Medical Research, Kuala Lumpur, Malaysia for more than 30 years unexposed to any infective agent. Mosquitoes were maintained at 70-80% relative humidity, 24-25°C and 12h:12h photoperiod.

Dengue and chikungunya virus

Dengue virus was maintained in the Institute for Medical Research, Kuala Lumpur, Malaysia. The virus was originally obtained from University of Malaya. Chikungunya virus was originally obtained from the Division of Virology, Nagasaki University, Japan. Both viruses were maintained in *Ae. albopictus* C6/36 cell line at 28°C in Eagle's minimum essential medium supplemented with 2% heat-activated fetal calf serum and 0.2 mM non-essential amino acids. The infected culture fluid was harvested 4-5 days after inoculation and cells were removed by sedimentation. The supernatant was then filtered through 0.22 µm filter unit (Nunc) and the viruses were concentrated using Integrated Speed Vac system (Savant ISS 100SC) at 14,000 rpm for 3 hours. Dengue and chikungunya viruses ranging between 8.1 - 8.5 x 10^5 pfu were used for artificial membrane feeding.

Artificial membrane feeding and transmission procedure

Artificial membrane feeding technique employed was modified from Graves (1980). Two hundred 4-7-day old female *Ae. aegypti* adults were starved overnight prior to artificial blood feeding. Approximately 30 female mosquitoes were placed into each paper cup. A glass feeder fitted with water jacket at 37°C was covered at the bottom by wrapping a small piece of membrane made from chicken skin moistened with normal saline. Fresh normal human blood was obtained by venipuncture on the day of blood feeding and immediately transferred into separate heparinized tube after which the blood was placed into the feeder. Mosquitoes were membrane-fed on a suspension containing 1 ml of human blood mixed with 100 µl of chikungunya and 100 µl of dengue viruses to obtain dual-infected sample. Uninfected sample was obtained by feeding mosquitoes with a suspension containing 1.0 ml of human blood and 200 µl of normal saline. Blood was presented to the mosquitoes by placing the cup containing mosquitoes below the feeder, with the surface of the nylon netting of the cup in contact with the membrane of the feeder. Each cup of mosquitoes was allowed to feed for approximately 10 to 30 minutes.

After feeding, all the mosquitoes in each cup were transferred into a cage. Only fully engorged mosquitoes were collected and were maintained for 7 days on 10% sucrose solution enriched with 1% vitamin B complex. Mosquitoes were transferred into sterile

eppendorf tubes (single or 10 individuals per tube) and kept at -20ºC for further use. All infectious studies were conducted in an isolated infection room.

Detection of chikungunya and dengue viruses using reverse transcriptase polymerase chain reaction (RT-PCR)

Single or mosquito pool was homogenized and RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen). For positive control, an equal volume of cultured cells separately infected with dengue and chikungunya virus was used and for negative control, uninfected cultured cells were used. Extracted RNA was kept at -20ºC until used.

RT-PCR method of Hasebe *et al* (2002) was employed. Extracted RNA was separated equally for dengue and chikungunya virus detection. Dengue virus consensus primers were 5'-TCAATATGCTGAAACG CGCAGAAACCG-3' and 5'-TTGCACC AACAGTCAATGTCTTCAGGTTC-3' (Lanciotti *et al*, 1992) and chikungunya virus primers 5'-TAGAGCAGGAAATT GATCCC-3' and 5'- CTTTAATCGCCTG GTGGTAT-3' (Hasebe *et al*, 2002). Master mix was prepared using Titan One Tube RT-PCR Kit (Roche). Each reaction contained 9.75 µl of double distilled water, 2 µl of dNTP mixture, 1.25 µl of dithiotreitol, 0.5 µl of RNAse inhibitor, 5.0 µl of RT-PCR buffer, 0.5 µl of enzyme mixture, 0.5 µl of each dengue or chikungumya primer and 0.5 µl of RNA.

For dengue virus detection, the reaction was carried out at 51ºC for 30 minutes to produce cDNA, which was then amplified by the following PCR steps : initial denaturation at 92ºC for 3 minutes, 41 cycles of 92ºC for 30 seconds, 51ºC for 45 seconds and 72ºC for 1 minute; followed by 72ºC for 5 minutes. For chikungunya virus detection, the reaction was carried out at 37ºC for one hour to produce cDNA, which was then amplified as follows: initial denaturation at 94ºC for 3 minutes; 35 cycles of 94ºC for 30 seconds, 54ºC for 90 seconds and 72ºC for 2 minutes; followed by 72ºC for 5 minutes. For every RT-PCR, a positive control and a negative control were included.

PCR products were analysed by performing electrophoresis in 2.0% Nusieve PCR gel (FC Bio, USA) at 100 volts and staining with ethidium bromide. The gel was viewed under ultraviolet illuminator (Ultra Lum, CA) and the resulting bands were photographed with a polaroid camera.

Minimum infection rate (MIR)

MIR was calculated as (number of positive pools or positive mosquitoes ÷ total number of mosquito tested) x 1,000.

RESULTS

Dengue and chikungunya infected culture fluid were confirmed positive using RT-PCR technique before carrying out artificial membrane feeding. The same procedure was carried for negative control. The 450 bp and 354 bp fragments were detected in dengue and chikungunya infected culture fluid, respectively.

Detection of dual infection in pooled mosquitoes

A total of 119 fully engorged female *Ae. aegypti* fed with both dengue and chikungunya virus were collected for dual infection study. In total, 117 individual mosquitoes survived and 12 pools were collected with 10 mosquitoes per pool. RNA was extracted from all pools and the extracted RNA samples were divided equally for RT-PCR amplification using universal dengue and specific chikungunya primers separately. The 450 bp dengue DNA was detected in 2 (pool number 13 and 14) of the pooled samples and the expected size of DNA product (354 bp) for chikungunya virus was

obtained in 3 different pooled samples (pool number 7, 8 and 9) (Fig 1). These data indicate the absence of dual infection.

Detection of dual infection in single mosquito

A total of 40 fully engorged female *Ae. aegypti* fed with both dengue and chikungunya virus were collected for dual infection study. Out of these, 36 individual mosquitoes survived and all mosquito samples were extracted individually and the extracted RNA samples were divided equally for RT-PCR amplification with universal dengue and specific chikungunya primers separately. The 450 bp and 354 bp amplicons were detected in 7 and 11 individual mosquitoes, respectively indicating the presence of only dengue or chikungunya virus in each mosquito (Fig 1).

MIR

When *Ae. aegypti* mosquitoes were infected with a mixture of both dengue and chikungunya viruses, MIR of pooled mosquitoes that demonstrated replication of dengue virus was 25, whereas the MIR of pooled mosquitoes that demonstrated replication of chikungunya virus was 17 (Table 1). MIR for single mosquitoes that were infected with dengue virus and chikungunya virus was 194 and 30, respectively (Table 1).

DISCUSSION

This study has shown that the expected 450 bp and 354 bp cDNA fragments were amplified from positive samples using universal dengue and chikungunya specific primers, respectively. Although only fully engorged *Ae. aegypti* were selected for the study, not all became infected. Weak bands were observed indicating that lower infection occurred in some of the mosquitoes tested. This may be due to the failure of the virus to replicate in the mosquitoes or that a few infected mosquitoes were present in the

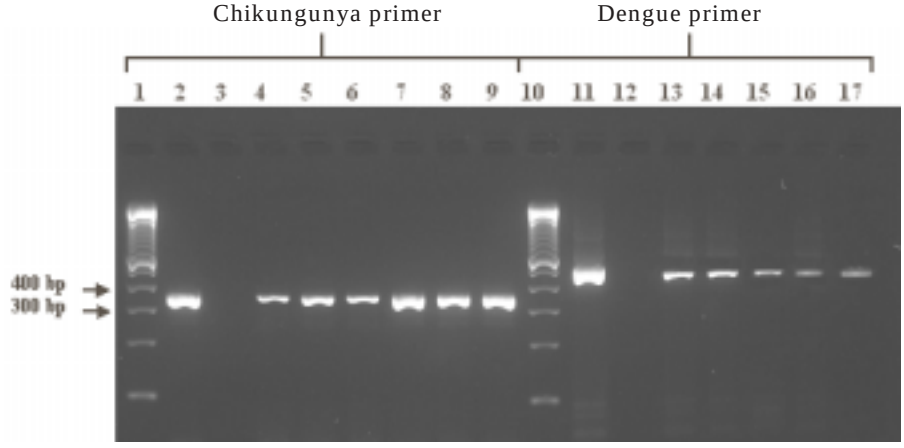

Fig 1–Detection of dengue and chikungunya viruses in *Ae. aegypti* by RT-PCR. Lanes 1 and 10: 100 bp DNA ladder marker; lane 2: positive control for chikungunya virus; lanes 3 and 12: negative control (uninfected mosquitoes); lanes 4, 5 and 6: chikungunya virus-positive samples from single infected *Ae. aegypti*; lanes 7, 8 and 9: chikungunya virus-positive samples from pool infected *Ae. aegypti*; lane 11: positive control for dengue virus; lanes 13 and 14: dengue virus-positive samples from pooled infected *Ae. aegypti*; lanes 15, 16 and 17: dengue positive samples from single infected *Ae. aegypti*.

Table 1
Minimum infection rate (MIR) of *Aedes aegypti* infected with chukungunya and dengue virus.

Sample	No. of mosquitoes	No. of pools tested	No. of positive pool	% Positive pool	MIR
Pooled specimen					
Negative control	64	7	0	0	0
Mixed feeding	117	12	2 (dengue)	17	17
			3 (chikungunya)	25	26
Single mosquito					
Negative control	30	15	0	0	0
Mixed feeding	40	36	7 (dengue)	19	194
			11 (chikungunya)	30	305

pools. A higher percentage of mosquitoes could be infected orally by increasing the virus dosage. Urdaneta *et al* (2005) reported that there is individual variation among mosquitoes of a given strain, not only in susceptibility to oral infection but also in ability to support virus replication.

The studies demonstrated that both dengue and chikungunya virus could not multiply in the same mosquito simultaneously. More chikungunya viruses were detected from the mosquito samples tested compared to dengue virus. Chikungunya primers showed consistently stronger PCR bands among positive samples as compared with dengue positive mosquito samples when amplified with universal dengue primers. This indicated that the rate of multiplication of chikungunya virus was faster than dengue virus in this vector species. This can be explained by the different incubation periods of these viruses in mosquito. Mourya *et al* (2001) reported that the multiplication of certain chikungunya virus strains in orally infected *Ae. aegypti* is robust and that the mosquitoes could transmit the virus on Day 4 post-infection. In comparison to chikungunya virus, the rate of multiplication of dengue virus is slow in this vector species ranging from 8 to 14 days post-infection (Mourya and Yadav, 2006). It may be assumed that if there is dual infection in *Ae. aegypti* mosquitoes, chikungunya virus would replicate and disseminate more rapidly, generating a situation where the detection of dengue virus could be difficult.

At this stage it is difficult to postulate that one virus may suppress replication of the other in dually fed mosquitoes. Although it is not known if transmission occurs through two different mosquitoes or through one double-infected mosquito, cases of simultaneous human infection with chikungunya and dengue-2 viruses have been reported (Mourya *et al*, 2001). More work is needed to demonstrate the dual infection of *Ae.aegypti* with both dengue and chikungunya viruses. Detecting virus infection every day after post-feeding should be conducted because both viruses have different incubation periods. Intrathoracic inocculation of *Ae. aegypti* with both dengue and chukungunya virus may also show more accurate results.

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