RESEARCH NOTE

COMPETITIVE SUPPRESSION BETWEEN CHIKUNGUNYA AND DENGUE VIRUS IN *AEDES ALBOPICTUS* C6/36 CELL LINE

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Abstract. *Aedes albopictus* C6/36 cell line was used to evaluate dengue virus serotype-3 (DENV-3) and chikungunya virus (CHIKV) co-infection. Virus infection was determined using a one-step duplex reverse transcriptase polymerase chain reaction (D- RT-PCR). D-RT-PCR was positive for both viruses when equal multiplicity of infection (MOI) was utilized. Co-infection with different titers between a higher CHIKV titer (MOI = 1.0) than DENV-3 titer (MOI = 0.1) showed similar results with that of equal titer. However, co-infection with a lower CHIKV titer (MOI = 0.1) than DENV-3 titer (MOI = 1.0) revealed positive results for only dengue virus infection, suggesting DENV competitive suppression of CHIKV. This competitive suppression occurred in mixed-infection samples but not in individually double infection (super infection) samples which produced both dengue and chikungunya virus progenies. Both virus replications depend on the amount of virus titer rather than serial infection. These findings have provided information regarding pathogen-pathogen interaction in host cell, which could be used to predict outbreaks, and to develop virus detection and vector control.

Keywords: chikungunya virus, dengue virus, *Aedes albopictus* C6/36 cell line, competitive suppression

INTRODUCTION

Dengue virus (DENV) and chikungunya virus (CHIKV) are medically important viruses causing morbidity among millions of people worldwide (Thaikruea *et al*, 1997; Anantapreecha *et al*, 2005; Dash *et al*, 2007; Dehecq *et al*, 2010). Both viruses cause public health problems in Southeast Asia. There have been outbreaks of these viruses reported from India, Singapore, Malaysia and Thailand (Simon *et al*, 2008; Yamamoto *et al*, 2010; Ho *et al*, 2011). The first reported case of

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chikungunya fever in Thailand occurred in 1954 caused by the Asian chikungunya genotype (Halstead *et al*, 1969a,b). The main vector of CHIKV is *Aedes aegypti* (Thavara *et al*, 2009). The outbreaks of chikungunya fever in Southeast Asia in 2008-2009 were related to the East, Central and South African genotype (ECSA), which is transmitted by *Ae. albopictus* (Chastel, 2005; Auksornkitti *et al*, 2010). The ECSA genotype is closely related to viruses isolated from earlier outbreaks in Malaysia and Singapore (Rianthavorn *et al*, 2010a). CHIKV has mutation A226V, which allows more case transmission by *Ae. albopictus* than *Ae. aegypti* (Bessaud *et al*, 2006; D'Ortenzio *et al*, 2011).

DENV infection is a public health problem in Thailand, with the number of cases rising to 56,651 and 50 deaths in 2009, a 2.23 fold increase from the previous year (Ministry of Public Health, 2009). Since 2009, CHIKV infections have occurred in Phuket, Songkhla, Pattani, Narathiwat, and Yala Provinces of Thailand with 24,029 cases reported (Hammon *et al*, 1960; Barbazan *et al*, 2002; Pongsiri *et al* 2010; Rianthavorn *et al*, 2010a,b). Symptoms of chikungunya fever are similar to dengue fever such as prolonged joint pain, swelling of joints, stiffness, muscle pain, headache, fatigue, nausea, vomiting, and rashes (Pardigon, 2009; Rianthavorn *et al*, 2010b). Detection and proper treatment are very important especially in dengue-endemic areas.

In Thailand co-circulation of all four serotypes has been reported by the dengue epidemiological surveillance network (Thavara *et al*, 2006; Fried *et al*, 2010). After the initial large outbreaks of chikungunya fever in Thailand, co-circulation of all four dengue serotype are found every year (Fried *et al*, 2010; Tang *et al*, 2010; Zhao *et al*, 2010). Dengue virus serotype 1 (DENV-1) has been shown to be the primary infection and DENV-3 is a secondary infection (Thavara *et al*, 2006; Tang *et al*, 2010; Zhao *et al*, 2010). DENV-3 is the predominant infection among the Thai people, compared with the other serotypes, during the dengue outbreaks in Thailand, in 1962 (Nimmannitya *et al*, 1969). Additionally, DENV-3 was found in blood specimens and mosquito samples collected from a recent chikungunya outbreak in South Thailand (Raekiansyah *et al*, 2005; Thavara *et al*, 2006).

Co-infection of DENV and CHIKV in human sera has never been reported in Thailand, but it has been reported in other countries, such as India, Malaysia, and Germany (Nayar *et al*, 2007; Ezzedine *et al*, 2008; Chahar *et al*, 2009; Leroy *et al*, 2009; Schilling *et al*, 2009). Furthermore, CHIKV and DENV co-infection in mosquitoes was revealed in field population, and mixed infection among the four serotypes of dengue viruses has been demonstrated (Thavara *et al*, 2006; Mavale *et al*, 2010). Recent information has not been recorded regarding CHIKV and DENV co-infection. This study focused on characterization of DENV-3 and CHIKV co-infection *in vitro* using *Aedes albopictus* C6/36 cell line as host to determine the effects of DENV and CHIKV co-infection. Effect of co-infection between DENV and CHIKV in the mosquitoes would be valuable information to forecast the diseases outbreak and furthermore for disease control.

MATERIALS AND METHODS

Viral propagation and co-infection

Ae. albopictus mosquito C6/36 cell culture was maintained at 28ºC for virus propagation. CHIKV and DENV-3 were isolated from infected mosquitoes collected from southern Thailand. DENV-3

and CHIKV were propagated in a monolayer of C6/36 cells line and supernatant was used to calculate the viral titer via a plaque assay (Sakoonwatanyoo *et al*, 2006; Jarman *et al*, 2011). Experiments were divided into three groups: single virus infection, mixed infection (co-infection), and single virus superinfection. Equal volumes and MOIs of the two viruses were used in the single infection and superinfection groups. Superinfection group received two infection treatments, which involved non-infected cells being exposed to the first viral treatment for 1 hour, removing the virus with an acid glycine buffer, and exposing the cells to the second virus (Hung *et al*, 1999). Mixed infection used both equivalent and different titers. Experiments were done separately in triplicate replications.

Viral titer determination by standard plaque assay

LLC-MK_2 of Rhesus monkey kidney cell monolayer was grown in 6-well plate at a concentration 4 x 10^5 cell/well. DENV and CHIKV were diluted and used to infect LLC-MK_2 cells separately. Each well was covered with 1% methylcellulose and after 3-7 days cells were stained with 2% crystal violet. Plaque number were counted, and plaque performing unit (PFU/ml) calculated before applying MOI = 1 and 0.1 to other experiments. The viral titer was measured before and after infection, and the experiments were conducted separately in triplicate.

One-step duplex reverse transcriptase polymerase chain reaction (D-RT-PCR)

Viral RNA genome was extracted from infectious C6/36 cell culture fluid using Nucleospin® RNA II kit (Invitrogen, Carlsbad, CA). One-step RT-PCR was performed using SuperScript® III One-Step RT-PCR kit (Gibco/Invitrogen, Grand Island, NY) with Platinum® *Taq* DNA polymerase in a total volume 25 l, containing 100 ng of RNA template, 12.5 l of 2X Reaction mix buffer, 0.5 l of sense (2.5 M), 0.5 l of anti-sense (2.5 M) and 0.5 U SuperScript III RT/Platinum *Taq*. The specific primers were designed as previously described (Dash *et al*, 2008). The amplification program was as follows: reverse transcription at 55ºC for 30 minutes; a polymerase activation step at 94ºC, 2 minutes; 40 cycles of 30 seconds at 94ºC, 1 minute at 55ºC, and 72ºC for 10 minutes. The D-RT-PCR amplicons were analyzed by electrophoresis in 1.0% agarose gel and staining with ethidium bromide.

RESULTS

Determination of virus production from single infection and co-infection

D-RT-PCR was used to determine virus secreted into cell culture-fluid after 7 days post-infection. The CHIKV (ECSA) strain and DENV-3 were combined as a mixed infection of C6/36 cells. The results of mixed infections, with equal titers of each virus, showed both dengue and chikungunya virus infection in the same culture supernatants (Fig 1). The result indicated that the two viruses were able to replicate in mosquito cell line. We also evaluated mixed infection using unequal titer of the two viruses at MOIs of 0.1 and 10. When a lower DENV-3 titer (MOI = 0.1) than CHIKV (MOI = 1.0) titer was used, D-RT-PCR results showed infection of two viruses, which was similar to co-infection with equal titers (Fig 1, lane 8). Unexpectedly, when we used a higher titer of DENV-3 (MOI = 1.0) than CHIKV (MOI = 0.1) for mixed-infection, D-RT-PCR produced an amplicon of 490 bp of DENV-3 *C-prM* gene only, while the CHIKV amplicon was not present (Fig 1, lane 7).

Determination of virus production from super infection of C6/36 cells

In one treatment group, C6/36 cells were first exposed to DENV-3, followed by CHIKV (in equal titer). D-RT-PCR results showed the presence of both viruses in the same culture fluid (Fig 1, lane 9). Reversal in the order of virus exposure yielded the same results (Fig 1, lane 10). Increasing the viral titer 10-fold from MOI = 1.0 to 10.0 gave results similar to that using equal titer (data not shown). These findings indicated that dengue and chikungunya viruses were able to co-infect C6/36 cells, no matter the order of infection, with only one exception, namely, co-infection with a lower CHIKV titer than DENV-3 which results in inhibition of CHIKV. These data demonstrate that DENV-3 and CHIKV infections depend on the amount of virus titer and not infection order. Thus, the two viruses can potentially infect host cells concurrently, as long as the viral concentration is high enough.

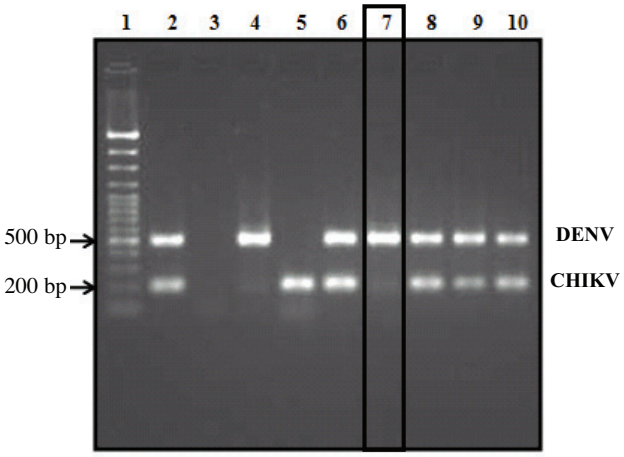

Fig 1–The positive results of mixed dengue and chikungunya infection evaluated by D-RT-PCR. All samples were separated by 1% agarose gel electrophoresis and stained with ethidium bromide. Lane 1, 100 bp DNA ladder (Fermentus); lane 2, positive control of dengue and chikungunya virus; lane 3, negative control; lane 4, single infection with DENV-3 virus; lane 5, single infection with CHIKV; lane 6, mixed infection, with equal titer of DENV-3 and CHIKV; lane 7, mixed infection, with higher dengue-virus titer than CHIKV; lane 8, mixed infection with lower dengue-virus titer than CHIKV; lane 9, super infection with DENV-3 following CHIKV; lane 10, super infection with CHIKV following DENV-3.

DISCUSSION

Our results are the first report that illustrated co-infection between DENV-3 and CHIKV (ESCA strain) derived from field caught mosquito vectors in Thailand. We demonstrated that single infection of DENV-3 or CHIKV in C6/36 cell line had 80% cytopathic effect (CPE) (data not shown), and the virus production are increased until 7 days post-infection similar to previous report (Sakoonwatanyoo *et al*, 2006). Mixed infection of DENV-3 and CHIKV with equal or unequal titers demonstrated both viruses were infective.

D-RT-PCR technique was used to determine virus production by detecting *C-prM* gene of DENV-3 and *E1* gene of CHIKV. The sensitivity of this assay has been shown to be better than the conventional virus isolation and could detect as low as 100 copies of genomic RNA (Dash *et al*, 2008). The benefit of this assay is the ability to detect dual infection of CHIK and DENV in one reaction.

Competitive suppression was seen in the sample of mixed infection when titer of DENV-3 (MOI = 1.0) was higher than that of CHIKV (MOI = 0.1). In contrast to experiments with equal titers of both viruses, or with lower titer of DENV-3 than CHIKV or super infection, both viruses were detected by D-RT-PCR in culture

fluid. These findings implied that DENV-3 has a high competitive infection so that it can suppress CHIKV replication. Sakoonwatanyoo *et al* (2006) have mentioned that specific receptors of DENV-3 are present in C6/36 cells enabling the virus to enter the insect cells. Competition between DENV-3 and CHIKV in our experiments could be related to the receptors on the insect cell (Mourya *et al*, 1998; Tio *et al*, 2005; Mercado-Curiel *et al*, 2006).

Asymmetric competitive suppression between two types of viruses (DENV-2 and DENV-4) was demonstrated in co-infection and super infection experiments in mosquito cell line (Hanley *et al*, 2008). These previous studies reported that infectious DENV-2 has higher suppression than DENV-4 in mixed infectious trials and were supposed to be due to intra-host competition among different strains of DENV. The competitive suppression in our experiment showing that DENV has higher concentration than CHIKV might help explain how reemergence outbreak between DENV and CHIKV occurred in co-circulation area. DENV and CHIKV are transmitted by the same mosquito species and viral transmissions are related to vector competence and extrinsic or intrinsic factors in mosquito (Beerntsen *et al*, 2000). One of the extrinsic factors is the viral concentration, and when mosquitoes are infected by two viruses at the same time, such as CHIKV and DENV, as the latter has a higher concentration, it could be able to suppress CHIKV replication in the mosquitoes. The intrinsic factors of mosquito vectors competence relate to specific receptors for the virus to enter the mosquito cells.

Our findings might be applied to predict the evolutionary epidemiology of medically important viruses. The competitive suppression is related to decreased virus transmission while mixed infection and super infection experiments supported the notion of two virus co-circulations in endemic area (Hanley *et al*, 2008). Further studies are required to determine how to control and treat chikungunya and dengue virus infections among the Thai population.

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