Evaluation of Simultaneous Transmission of Chikungunya Virus and Dengue Virus Type 2 in Infected *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae)

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J. Med. Entomol. 52(3): 447–451 (2015); DOI: 10.1093/jme/tjv017

ABSTRACT The simultaneous transmission of chikungunya virus (CHIKV) and dengue viruses (DENV) has been a major public health concern because of their sympatric distribution and shared mosquito vectors. Groups of *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse) were orally infected with 1.5×10^5 PFU/ml of CHIKV and 3.2×10^6 FFU/ml of DENV-2 simultaneously or separately in inverse orders and evaluated for dissemination and transmission by qRT-PCR. Simultaneous dissemination of both viruses was detected for all groups in *Ae. aegypti* and *Ae. albopictus* while cotransmission of CHIKV and DENV-2 only occurred at low rates after sequential but not simultaneous infection.

KEY WORDS *Aedes aegypti*, *Aedes albopictus*, chikungunya, dengue, transmission

The rapid spread of chikungunya virus (CHIKV) has led to its cocirculation with dengue viruses (DENV) in many tropical or subtropical regions (Hertz et al. 2012, Singh et al. 2012). The first report of a patient coinfected with both CHIKV and DENV occurred in Vellore, South India, in 1967 (Myers and Carey 1967). Following the 2005–2007 outbreak of CHIKV, more contemporary occurrences of dual CHIKV and DENV infections have been identified in numerous patients from India, Sri Lanka, Malaysia, and Gabon (Chahar et al. 2009, Leroy et al. 2009). The observation of dual infection has not been limited to patients that reside within these endemic regions. More recently, travelers to areas where CHIKV and DENV cocirculate are becoming infected with both viruses (Schilling et al. 2009, Chang et al. 2010). The establishment of local transmission of CHIKV in the Caribbean islands, where DENV has been endemic for nearly two decades, has further increased the importance of evaluating the possibility of concurrent CHIKV and DENV infection in *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse). Human coinfection of CHIKV and DENV could result from successive exposure to mosquitoes infected with either CHIKV or DENV or exposure to mosquitoes dually infected with both viruses. This study addresses the latter possibility. The principle vector for both of these viruses is the *Ae. aegypti* mosquito (Mourya and Yadav 2006). *Ae. albopictus* is an efficient vector of both CHIKV and DENV in the past decade and exceeds the CHIKV vectoring capacity of *Ae. aegypti* in cooler geographical regions where the virus is not endemic or where certain CHIKV strains circulate (Lounibos 2002; Braks et al. 2003; Juliano and Lounibos 2005; Simard et al. 2005; Bagny et al. 2009a,b; Kamgang et al. 2010; Paupy et al. 2010).

Despite increased interest in the detection of dual CHIKV and DENV infections in humans and vectors, the limited availability of molecular epidemiological tools in the field complicates identification of these events. Thus, assessing the susceptibility of mosquitoes or mosquito cells is largely based on laboratory experimental conditions and *in vitro* studies (Zebovitz and Brown 1968, Stollar and Shenk 1973, Johnston et al. 1974, Eaton 1979, Karpf et al. 1997). Studies of dual infection of CHIKV, a member of the genus of *Alphavirus* in the family *Togaviridae*, and DENV-2, a *Flavivirus* in the *Flaviviridae* family, in mosquitoes can be complicated by the sequence of exposure, route(s) of challenge, and method(s) of viral quantification. Field-collected *Ae. albopictus* are susceptible to coinfection with CHIKV and DENV-2 when the viruses are presented in separated artificial blood meals (Moutailler

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et al. 2009). In addition, simultaneous transmission of CHIKV and DENV-1 by *Ae. albopictus* was observed after oral infection with CHIKV and intrathoracic inoculation of DENV-1 (Vazeille et al. 2010). Rohani found that per os exposure of *Ae. aegypti* mosquitoes to artificial blood meals containing both CHIKV and DENV-2 led to the heterologous exclusion between either of the viruses (Rohani et al. 2009).

The objective of our experiments was to determine the vector transmission capabilities of CHIKV and DENV-2 in *Ae. albopictus* and *Ae. aegypti* mosquitoes after different sequences of exposures to both viruses. To our knowledge, this is the first report assessing the impact of the different sequence of exposure to CHIKV and DENV-2 on viral dissemination and transmission by *Ae. aegypti* and *Ae. albopictus* and describing the concurrent secretion of CHIKV and DENV-2 in the saliva of infected mosquitoes.

Materials and Methods

Mosquitoes and Viruses. Generation F≥5 female *Ae. aegypti* Higgs white-eye strain and *Ae. albopictus* La Réunion were maintained in an arthropod containment level-2 insectary in accordance with standard rearing practices at 28°C with a photoperiod of 16:8 (L:D) h, as previously described (Vanlandingham et al. 2006, Nuckols et al. 2013). Anesthetized hamsters were provided on a weekly basis for hematophagous stimulation of vitellogenesis and subsequent oviposition on moistened paper towels for sustainment of the colonies under Protocol number 0003019 approved by the University of Texas Medical Branch (UTMB) Institutional Animal Care and Use Committee. Mosquitoes were infected with CHIKV produced from the CHIKV La Réunion infectious clone pCHIKV-LR i.c. electroporated in BHK-21 cells, as previously described (Tsetsarkin et al. 2006). The DENV-2 New Guinea C strain was prepared by infecting C6/36 (*Ae. albopictus*) cells in Dulbecco's modified Eagle's medium (high-glucose DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 10% tryptose phosphate broth, 1% L-glutamine, and 1% 100× penicillin–streptomycin at 28°C with 5% CO_2. CHIKV and DENV-2 were titrated by plaque assay on Vero cells maintained in Liebovitz L-15 media (L-15; Invitrogen), as previously described (McElroy et al. 2006, Tsetsarkin et al. 2006, Nuckols et al. 2013). To enhance the visualization of DENV-2 viral plaques, immunostaining with anti-DENV 4G2 monoclonal antibody was performed with the focus-forming assay previously published (McElroy et al. 2006). The use of CHIKV and DENV-2 was approved by UTMB Institutional Biosafety Committee.

Oral Infection of Mosquitoes. Mosquitoes were orally infected with 1.5×10^7 PFU/ml of CHIKV and/or 3.2×10^6 FFU/ml DENV-2 mixed with defibrinated sheep blood (DSB; Colorado Serum Company, Denver, CO) simultaneously (1:1:1 CHIKV:DENV-2:DSB) or separately (1:1 virus:DSB) using a Hemotek arthropod feeder (Discovery Workshops, Accrington, Lancashire, United Kingdom) in an arthropod containment level-3 insectary, as described previously (Tsetsarkin et al. 2006, Vanlandingham et al. 2006). Engorged mosquitoes were sorted and returned to cartons and damp paper towels in a 3-ml cup with water were placed in cartons to allow for oviposition in advance of the second per os infection. The cartons were placed in humidified containers maintained at 28°C with a photoperiod of 16:8 (L:D) h and offered wicked 10% sucrose water up to 17 d postinfection before collection of tissues and saliva.

Virus Detection. The evaluation of coinfection with CHIKV and DENV by *in vitro* infectious assay is technically difficult owing to the more rapid replication and cytopathogenicity of CHIKV compared with DENV. Therefore, we used an optimized qRT-PCR (Assay Development Services Division of the Galveston National Laboratory, UTMB) to directly detect virus in mosquito heads for dissemination and in saliva to evaluate transmission potential, as described previously (Vanlandingham et al. 2013). At 17 d postinfection (dpi), saliva was collected by inserting each mosquito's proboscis into 50-µl capillary tubes (Drummond Scientific Co., Broomall, PA) containing ~10 µl of type B immersion oil (Cargille Laboratories Inc., Cedar Grove, NJ). After 1 h, the contents of the capillary tubes with visually discernible saliva secretion were dispensed into microcentrifuge tubes containing 200 µl of β-mercaptoethanol (BME)-supplemented Aurum lysis solution, while the corresponding heads were placed into another microcentrifuge tube containing 100 µl of BME-supplemented Aurum lysis solution. RNA extraction for qRT-PCR analysis was performed using the Aurum Total RNA 96 kit, according to the manufacturer's protocol. While maintained on ice, an equal amount of 70% ethanol with the RNase inhibitor diethylpyrocarbonate, was added to each sample (head = 100 µl and saliva = 200 µl), and samples were transferred to an Aurum total RNA binding plate in 150-µl aliquots and centrifuged for 2 min at 4,000 rpm. Viral RNA of CHIKV and DENV-2 were reverse transcribed with iScript reverse transcriptase (BioRad, Hercules, CA) and quantified by iQ5 SYBR Green Supermix (BioRad, Hercules, CA) and iQ Supermix (BioRad, Hercules, CA), respectively. CHIKV was amplified using the following primers: forward 5′-TCCTGACCACCCAACACTCCTG-3′ and reverse 5′-ATACTTATACGGCTCGTTG-3′. DENV-2 was amplified using DENV-2 fluorescent probe 5′-HEX-CTGTCTCCTCAGCATCATTCCAGGCA-BHQ1-3′ and forward and reverse primers; forward 5′-CATATTGACGCTGGGAAAGA-3′ and reverse 5′-CATTC CATTTTCTGGCGTTCT-3′.

Statistical Analysis. Normalized quantitative genomic copies of CHIKV and/or DENV-2 in collected mosquito heads and non-normalized saliva samples were analyzed in both *Ae. albopictus* and *Ae. aegypti* mosquitoes by two-way analysis of variance in SPSS Statistical Software. Statistically significant differences in dissemination and transmission rates between groups were identified by Fisher's exact test in R statistical software (significance at $p < 0.05$).

Table 1. Dissemination of CHIKV and DENV-2 17 d.p.i. in *Ae. aegypti* and *Ae. albopictus*

Species	Sequence of exposure	Dissemination		
		CHIKV (%)	DENV (%)	CHIKV and DENV (%)
Ae. aegypti	CHIKV	5/8 (62.5)	–	–
	DENV	–	8/20 (40)	–
	CHIKV-4dpi-DENV	9/9 (100)	3/9 (33.3)	3/9 (33.3)
	DENV-8dpi-CHIKV	24/26 (92.3)	4/26 (15.9)	4/26 (15.9)
	CHIKV + DENV	14/29 (48.3)	4/29 (13.8)	2/29 (6.9)
Ae. albopictus	CHIKV	13/13 (100)	–	–
	DENV	–	4/10 (40)	–
	CHIKV-4dpi-DENV	16/16 (100)	6/16 (37.5)	6/16 (37.5)
	DENV-8dpi-CHIKV	22/25 (88)	16/25 (64)	16/25 (64)
	CHIKV + DENV	22/26 (84.6)	9/26 (34.6)	8/26 (30.8)

Results and Discussion

Experiments were grouped as follows: 1) CHIKV-4dpi-DENV-2, CHIKV-exposed mosquitoes subsequently offered DENV-2 at 4 dpi; 2) DENV-2-8dpi-CHIKV, DENV-2-exposed mosquitoes subsequently offered CHIKV at 8 dpi; 3) CHIKV + DENV-2, mosquitoes simultaneously exposed to CHIKV and DENV-2; 4) CHIKV-4 dpi-DSB, CHIKV-exposed mosquitoes subsequently offered DSB; and 5) DENV-2-8 dpi-DSB, DENV-2-exposed mosquitoes subsequently offered DSB. These infection schedules were chosen based on previous studies of growth kinetics for each virus, in which CHIKV presented peak whole mosquito titers between 3 and 4 dpi, whereas DENV-2 presented peak titers between 7 and 8 dpi. Analysis of CHIKV in the heads of *Ae. aegypti* mosquitoes compared with CHIKV in the heads of *Ae. albopictus* mosquitoes between the three dual infection techniques found the number of CHIKV genomic copies to be significantly higher ($t = 3.8302$; $df = 105$; $P = 0.0002$) in the *Ae. albopictus* mosquitoes. This is consistent with the previous observation that *Ae. albopictus* were more permissive to CHIKV dissemination (Tsetsarkin et al. 2007). There was no statistically significant difference among the three routes of dual infection (data not shown). Comparison of the number of genomic copies of DENV-2 in the heads of *Ae. albopictus* and *Ae. aegypti* mosquitoes between the three infection schemes found no statistically significant difference between both species or order of virus exposure. Similarly, no significant differences in CHIKV or DENV-2 genomic copy number were detected in saliva collected from *Ae. albopictus* or *Ae. aegypti* mosquitoes in any experimental group (data not shown).

The dissemination and transmission rates for all experimental groups are presented in Tables 1 and 2, respectively. We demonstrated that 30.8% of *Ae. albopictus* and 6.9% of *Ae. Aegypti* collected at 17 dpi developed a disseminated infection with CHIKV and DENV-2 after simultaneous exposure to both viruses, but simultaneous CHIKV and DENV-2 transmission was not detected in either mosquito after simultaneous infection. This suggests that competition between simultaneously introduced viruses may decrease the transmission potential of both when compared with sequential infection due to a potential bottleneck or another, as yet unidentified, mechanism.

Simultaneous CHIKV and DENV-2 dissemination and transmission were detected in both mosquito species after sequential infection. In *Ae. albopictus*, a significantly higher rate of dual dissemination ($P < 0.05$) occurred in mosquitoes infected with DENV-2 followed by CHIKV(64%) compared with those infected with the inverse order (37.5%) or simultaneously (34.6%). Detection of CHIKV and DENV-2 in saliva occurred in both groups of *Ae. albopictus* infected sequentially; however, significant difference was not observed (37.5 vs. 16.7%, $P > 0.05$). Dual dissemination was detected in 15.9% of *Ae. aegypti* infected with DENV-2 then CHIKV and in 33.3% of *Ae. aegypti* infected with CHIKV followed by DENV-2, but dual virus transmission only occurred in one mosquito infected with CHIKV followed by DENV-2 (33.3%).

CHIKV consistently demonstrated higher rates of dissemination than DENV-2 in all infection schemes ($P < 0.05$). This could be a function of greater susceptibility of *Ae. aegypti* and *Ae. albopictus* to CHIKV than to DENV-2. Alternatively, the more robust propagation of CHIKV resulted in a significantly higher CHIKV dissemination rate than DENV, although the 17-d-long incubation period would have exceeded the time required for both DENV and CHIKV to replicate above the limit of detection of the assay in this study. There was no significant difference in simultaneous transmission of DENV-2 and CHIKV between *Ae. albopictus* fed DENV-2 then CHIKV and *Ae. albopictus* fed CHIKV then DENV-2, suggesting that sequence of infection may not interfere with dual transmission by *Ae. albopictus*, although the same was not observed for *Ae. aegypti*.

The ability of CHIKV and DENV-2 to be dually transmitted by *Ae. aegypti* following sequential infection conditions, as opposed to the simultaneous infection of both CHIKV and DENV-2, was observed, which was consistent with a previous report that described the lack of dual infection after the simultaneous per os challenge of DENV and CHIKV (Rohani et al. 2009). Additional work will be required to determine the mechanism underlying this observation.

Table 2. Transmission of CHIKV and DENV-2 17 d.p.i. in *Ae. aegypti* and *Ae. albopictus*

Species	Sequence of exposure	Transmission		
		CHIKV (%)	DENV (%)	CHIKV and DENV (%)
Ae. aegypti	CHIKV	3/5 (60.0)	–	–
	DENV	–	6/8 (75.0)	–
	CHIKV-4dpi-DENV	3/9 (33.3)	2/3 (66.7)	1/3 (33.3)
	DENV-8dpi-CHIKV	10/24 (41.7)	1/4 (25.0)	0/4 (0.0)
	CHIKV + DENV	5/14 (35.7)	2/4 (50.0)	0/2 (0.0)
Ae. albopictus	CHIKV	7/13 (53.8)	–	–
	DENV	–	2/4 (50.0)	–
	CHIKV-4dpi-DENV	8/16 (50.0)	1/6 (16.7)	1/6 (16.7)
	DENV-8dpi-CHIKV	11/22 (50.0)	11/16 (68.8)	6/16 (37.5)
	CHIKV + DENV	7/22 (31.8)	1/9 (11.1)	0/8 (0.0)

Despite the relatively small sample size tested, our results clearly provide evidence that the simultaneous infection, dissemination and transmission of CHIKV and DENV-2 can occur, especially by orally administering two viruses separately. It is possible that immune responses to primary infection facilitate secretion of both CHIKV and DENV-2 in saliva following a sequential infection. Recent work by Mousson et al. (2012) suggested that the presence of *Wolbachia* might restrict the viral density of DENV in *Ae. albopictus* salivary glands, and van den Hurk et al. (2012) found decreased CHIKV infection and dissemination in *Wolbachia*-infected *Ae. aegypti*. We observed a non-significant increase in DENV-2 transmission in *Ae. albopictus* infected secondarily with CHIKV, suggesting that the dynamics underlying pathogen coinfection of mosquitoes warrants further investigation. As reported by Sim et al. (2010), it is likely that the establishment of infection by arboviruses can suppress the innate immune responses of infected mosquitoes. Therefore, the enhancement of DENV-2 transmission by the subsequent CHIKV infection may also be the consequence of the suppression of innate immune responses of infected mosquitoes by CHIKV. Further investigation of these mechanisms is indicated for future studies, particularly by introducing the second virus at different time points. This factor was not incorporated into our experimental design, which was initially constructed based on the propagation and incubation periods of CHIKV and DENV-2 in vitro and in mosquitoes.

Future work on this topic should focus on sequential infection events of both viruses in two different wild-caught vector species, as this produced the highest likelihood of dual infection. In addition, further evaluation of potential interference mechanisms that reduce CHIKV and DENV dissemination and transmission would enhance the understanding of heterologous interference events that may exist between alphaviruses and flaviviruses and potential requirements for the occurrence of cocirculation of both viruses in nature.

Acknowledgments

We wish to thank Jing Huang for her invaluable knowledge of mosquito manipulation and her persistent efforts to assist in the administration of enemas to the mosquitoes. This work was in part supported by National Institutes of Health (NIH) grant R21 A1073389 (to S.H.) and J.T.N. was supported by the U.S. Army Medical Department's Long Term Healthcare Education and Training program.

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Received 4 June 2014; accepted 22 January 2015.