

Susceptibility of Florida Mosquitoes to Infection with Chikungunya Virus

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Abstract. Chikungunya virus (CHIKV) has caused recent, large epidemics on islands in the Indian Ocean, raising the possibility of more widespread CHIKV epidemics. Historically, CHIKV has been vectored by *Aedes aegypti*, but these outbreaks likely also involved *Ae. albopictus*. To examine the potential for an outbreak of CHIKV in Florida, we determined the susceptibility to CHIKV of F₁ *Ae. aegypti* and *Ae. albopictus* from Florida. In addition, we also evaluated two well-characterized laboratory strains (Rockefeller and Lake Charles) of these species. We determined infection and dissemination rates as well as total body titer of mosquitoes 7 days post-exposure (pe) (*Ae. albopictus*) and 3, 7, and 10 days pe (*Ae. aegypti*). All mosquito strains were susceptible to both infection and dissemination, with some variation between strains. Our results suggest Florida would be vulnerable to transmission of CHIKV in urban and rural areas where the two vector species occur.

INTRODUCTION

The 2004–2007 outbreak of chikungunya virus (CHIKV) started in Kenya and spanned the countries bordering the Indian Ocean and caused illness in more than 250,000 persons on La Réunion island, and possibly more than a million in India.^{1–4} Although isolated in 1952,⁵ CHIKV has received little attention because of the low mortality associated with it and the limited extent of previous epidemics relative to the current outbreak. This virus has a sylvatic cycle in Africa between wild primates and forest *Aedes* mosquitoes, while in Asia and the Indian Ocean it cycles exclusively between humans and *Aedes* spp. mosquitoes.⁶ The large scale of the Indian Ocean outbreak, and increased frequency of epidemics throughout south Asia suggests the possibility CHIKV could emerge in non-endemic areas, such as the United States.^{7,8} Numerous imported cases of CHIKV to the United States and Europe have been recently reported.^{9–12}

The two principal vectors of CHIKV in the Indian Ocean basin are *Aedes aegypti* and *Ae. albopictus*.^{13–15} *Aedes aegypti* has been established in North America for probably more than 300 years,¹⁶ and since 1985 the southeastern United States has been invaded by *Ae. albopictus*, whose distribution now ranges from Illinois to Florida.^{17–19} *Aedes aegypti* decreased in range and abundance concomitant with this invasion and is currently limited to urban settings in Florida and other southern states, where it may be locally abundant.^{17,20} Determining the ability of local populations of these two mosquito species to acquire and transmit the outbreak strain of CHIKV is an important step for considering public health responses in the event of an outbreak.

Previous studies have found variation in the susceptibility of mosquito species and strains to CHIKV. An early study found *Ae. aegypti* the most refractory of five mosquito species to infection, half as susceptible as *Ae. albopictus*.²¹ Tesh and others,²² working with two strains of CHIKV, reported large vector competence variation between several strains of *Ae. albopictus*. In a more recent study, *Ae. albopictus* was also more likely than *Ae. aegypti* to become infected and develop a disseminated infection with a strain of CHIKV isolated

from an outbreak in Bangkok, Thailand in 1962.²³ This study also found significant variation between strains of *Ae. albopictus*.

Recent investigations have suggested that the 2005 La Réunion outbreak may have been caused by an increased transmission of CHIKV by *Ae. albopictus*, making the variation in susceptibility of mosquitoes to this strain of CHIKV a timely and pertinent question.^{24,25} Furthermore, the size of the ongoing outbreak in the Indian Ocean region and the widespread invasion by *Ae. albopictus* of the United States increase the risk of introduction and transmission of CHIKV in North America. For these reasons, we tested the susceptibility of current Florida and well-characterized laboratory strains of *Ae. aegypti* and *Ae. albopictus* to a La Réunion isolate of CHIKV.

MATERIALS AND METHODS

Mosquitoes. Four mosquito strains were tested in this experiment: F₁ *Ae. aegypti* from F₀ field collections made from December 2006 to January 2007 in Palm Beach County (added biweekly for a total initial collection of approximately 100 female mosquitoes); F₁ *Ae. albopictus* from F₀ field collections made from December 2006 to January 2007 in Palm Beach County (added biweekly for a total initial collection of approximately 250 female mosquitoes); Rockefeller strain *Ae. aegypti* maintained at the Florida Medical Entomology Laboratory (FMEL) since 2003; and Lake Charles strain *Ae. albopictus*, also maintained at FMEL since 2003. Both Lake Charles and Rockefeller strains have been in colony for many years, and their history has been described elsewhere.²⁶ Colonies were given 20% sucrose *ad libitum*, and blood fed on live chickens. Chicken care followed the animal use and care policies of the National Institutes of Health (University of Florida, IACUC Protocol VB-17). Colonies were kept in 14-hour light:10-hour dark cycle at 28 ± 1°C and a relative humidity > 80% at all times. To produce adults, eggs were hatched in tap water and put in groups of 50 in one liter of tap water and fed 0.3 g 1:1 yeast:albumin. Larvae were reared at 28°C with a 14-hour light:10-hour dark cycle.

Virus. We used CHIKV strain LR2006-OPY1, which was isolated from a febrile patient in France who had been infected in La Réunion,¹² then passaged once in African green monkey kidney (Vero) cells. The virus stock titer was determined (log₁₀ 7.2 plaque-forming units [pfu]/mL) by plaque assay as described previously.²⁷ Virus stocks were stored at

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–80°C. To produce fresh virus for mosquito infection, a T-75 tissue culture flask with a confluent monolayer of Vero cells in 10 mL of cell culture medium (M199 medium supplemented with 10% fetal bovine serum, antibiotics, and antimicrobials; Invitrogen, Carlsbad, CA) was inoculated with 100 µL of stock virus and incubated in a atmosphere of 5% CO₂ at 35°C for 24 hours.

Oral infection of mosquitoes. Mosquitoes were exposed to blood mixed with virus in artificial membrane feeders.²⁸ Three to six day-old mosquitoes were placed in cylindrical, waxed cardboard containers (height = 14 cm, diameter = 11 cm) (Dade Paper Co., Miami, FL) in groups of approximately 50 and placed in an incubator (14-hour light:10-hour dark cycle, with an automated dusk/dawn period of 1 hour at 28°C and a relative humidity of approximately 95%) within a certified Biosafety Level 3 containment facility. Sugar was removed 24 hours prior to feeding. Mosquitoes were exposed to blood meals consisting of a 1:10 dilution of freshly propagated CHIKV in citrated bovine blood (Hemostat Laboratories, Dixon, CA). Infectious blood was heated at 35°C for 20 minutes and placed on mosquito cages for one hour. After feeding, mosquitoes were cold anesthetized and only fully engorged mosquitoes were kept. Because of low feeding rates for *Ae. albopictus* (approximately 10%), female mosquitoes were exposed daily for three consecutive days. Samples of each blood meal were assayed using real-time, quantitative, reverse transcription–polymerase chain reaction (qRT-PCR) with copy number standardized to pfu by a plaque assay performed on 10-fold serial dilutions of virus stock.²⁹ Blood meal titers ranged between log₁₀ 5.9 pfu/mL and log₁₀ 6.5 pfu/mL, as measured by qRT-PCR.

Determination of infection kinetics and status. Blood fed *Ae. aegypti* females were killed (by freezing) 3, 7, and 10 days post-exposure (pe), and *Ae. albopictus* females at 7 days pe to check for infection and dissemination. Mosquito legs were separated from bodies and individual body and legs were triturated separately in 2-mL tubes with 900 µL of BA-1 media³⁰ and two zinc-plated BBs at 25 Hz for 3 minutes using a Tissuelyzer[®] tissue homogenizer (Qiagen Inc., Valencia, CA) and clarified by centrifugation (3,148 × g for 4 minutes). Samples were stored at –80°C until further processing no more than 10 days after freezing. Nucleic acid was then extracted using the MagNA Pure Instrument with the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Chicago, IL) following the manufacturer's instructions.

A one-step qRT-PCR was used to determine infection status and body titer of samples. Protocols have been described elsewhere.³¹ Primers were designed from the E1 gene (Genbank accession no. DQ443544) and had the following sequences: forward: 5'-ACC CGG TAA GAG CGA TGA ACT-3'; reverse: 5'-ACG CCG CAT CCG GTA TGT-3'. The probe sequence was: 5'-/5Cy5/CCG TAG GGA ACA TGC CCA TCT CCA /3BHQ_2/-3' (IDT DNA, Coralville, IA).

Statistical analysis. All statistical analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC). Proportion of mosquitoes infected (no. positive bodies/no. tested) or with disseminated infections (no. positive legs/no. positive bodies) were compared using contingency table analysis. If significant effects were detected, pairwise comparisons were made with an alpha adjusted to account for multiple comparisons ($\alpha = 0.05/n$, where $n =$ no. of comparisons). For strain comparisons, only data from 7 days pe were used because there

were insufficient mosquitoes from either *Ae. albopictus* group to harvest on days 3 or 10. Only body titers of mosquitoes with disseminated infections were compared. Transformations failed to make body titer data normally distributed, and rank tests (Kruskal-Wallis or Wilcoxon tests with a normal approximation) were used to detect significant variation in body titers between strains and between days pe for *Ae. aegypti* strains.³²

RESULTS

Effect of different mosquito strains: infection/dissemination. Mosquito strain significantly affected the proportion of mosquitoes infected ($\chi^2 = 10.867$, degrees of freedom [df] = 3, $P = 0.0125$; Table 1) and disseminated ($\chi^2 = 58.054$, df = 3, $P < 0.0001$; Table 1) on day 7 pe. The F₁ *Ae. aegypti* from Florida was significantly less likely to be disseminated than mosquitoes from other strains, and Rockefeller *Ae. aegypti* was significantly less likely to be disseminated than either strain of *Ae. albopictus*, with no difference between the two *Ae. albopictus* strains (adjusted $\alpha = 0.0083$). The only significant difference in infection status was between F₁ *Ae. aegypti* and Lake Charles *Ae. albopictus* ($\chi^2 = 9.4826$, df = 1, $P = 0.0021$).

Effect of different mosquito strains: body titer. Different mosquito strains had significantly different body titers for disseminated infections at day 7 pe ($\chi^2 = 61.47$, df = 3, $P < 0.0001$; Table 1). Lake Charles *Ae. albopictus* had significantly higher titers than the other three strains, between which there were no differences in body titer at day 7 pe (adjusted $\alpha = 0.0083$).

Effect of time for *Ae. aegypti* strains: infection/dissemination. Time since exposure significantly affected the proportion of disseminated infections among Rockefeller *Ae. aegypti* ($\chi^2 = 36.19$, df = 2, $P < 0.0001$; Figure 1), but not the proportion infected ($\chi^2 = 2.44$, df = 2, $P = 0.29$). The F₁ *Ae. aegypti* had a similar pattern, with a significant difference between days since exposure in dissemination ($\chi^2 = 50.31$, df = 2, $P < 0.0001$; Figure 1), but not infection ($\chi^2 = 1.051$, df = 2, $P = 0.59$). On days 3 and 7, but not day 10, there were significantly more Rockefeller *Ae. aegypti* with disseminated infections than F₁ *Ae. aegypti* when each day was compared separately (adjusted $\alpha = 0.0166$, day 3: $\chi^2 = 8.07$, df = 1, $P < 0.0045$; day 7: $\chi^2 = 13.53$, df = 1, $P < 0.0002$; day 10: $\chi^2 = 0.136$, df = 1, $P < 0.72$; Figure 1).

Effect of time for *Ae. aegypti* strains: body titer. Body titer varied significantly as a function of days since exposure for F₁ *Ae. aegypti* with disseminated infections ($\chi^2 = 25.94$, df = 2, $P < 0.0001$; Figure 2), but not Rockefeller *Ae. aegypti* ($\chi^2 = 1.18$, df = 2, $P < 0.55$; Figure 2). Body titer was significantly different on all days pe for F₁ *Ae. aegypti* (adjusted $\alpha =$

TABLE 1
Percent infected, percent disseminated, and body titer for mosquitoes seven days post-exposure to chikungunya virus for each strain tested

Mosquito strain	% Infected	% Disseminated	Mean ± SE log ₁₀ body titer*
F ₁ <i>Aedes aegypti</i>	93 (98/105)	67 (66/98)	5.63 ± 0.10
F ₁ <i>Ae. albopictus</i>	100 (29/29)	100 (29/29)	5.53 ± 0.08
Rockefeller <i>Ae. aegypti</i>	98 (76/77)	80 (69/76)	5.57 ± 0.11
Lake Charles <i>Ae. Albopictus</i>	100 (99/99)	98 (98/99)	6.21 ± 0.05

* Plaque-forming units/mL.

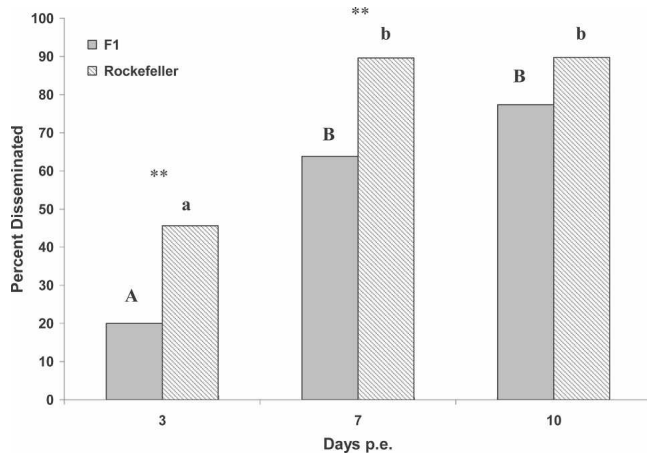


FIGURE 1. Percent disseminated versus days post-exposure for *Aedes aegypti* strains. **significant pairwise differences between strains ($\alpha = 0.0166$) by chi-square test. Letters denote homogenous groups by *post hoc* pairwise comparisons (uppercase for F₁ *Ae. aegypti*; lowercase for Rockefeller *Ae. aegypti*).

0.0166; Figure 2). On day 3 pe, but not day 7 pe or 10 pe, F₁ *Ae. aegypti* had significantly lower body titer than Rockefeller *Ae. aegypti* ($Z = -3.63$, $df = 1$, $P < 0.0003$).

DISCUSSION

All four mosquito strains tested were highly susceptible to infection with CHIKV. The F₁ *Ae. aegypti* from Florida was less susceptible, with slightly lower infection rates and significantly lower dissemination rates relative to Rockefeller *Ae. aegypti* and both *Ae. albopictus* strains. The course of infection in F₁ *Ae. aegypti* was slower than in the Rockefeller strain, and had a lower average body titer three days pe.

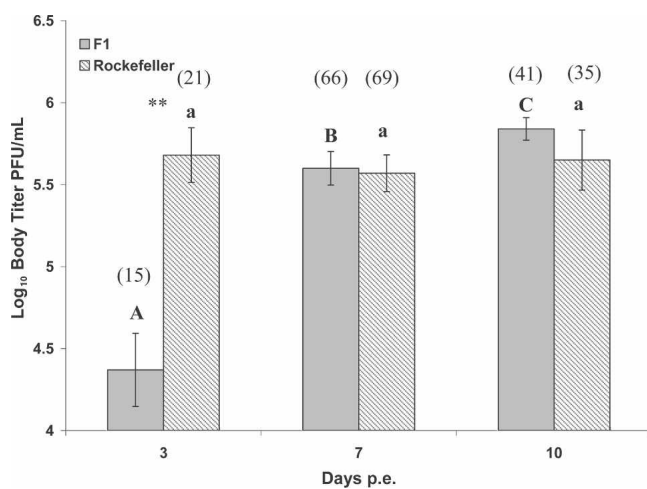


FIGURE 2. Body titer (mean \pm SE log₁₀ plaque-forming units/0.1 mL) for *Aedes aegypti* mosquitoes with disseminated infections. Letters denote homogenous groups comparing days post-exposure within strain by all possible pairwise comparisons using Wilcoxon's two-sample rank test ($\alpha = 0.0166$; uppercase for F₁ *Ae. aegypti*; lowercase for Rockefeller *Ae. aegypti*). **significant *post hoc* differences in body titer between strains by Wilcoxon's two-sample rank test ($\alpha = 0.0166$). Numbers in parentheses are the number of disseminated mosquitoes tested for each strain at each day.

There were no differences between the Rockefeller *Ae. aegypti* and the two *Ae. albopictus* strains in infection or dissemination rates, although Lake Charles *Ae. albopictus* had a higher average body titer than any other strain after seven days. Although the differences we observed were statistically significant, the biologic relevance of these differences is unknown.

The findings for *Ae. albopictus*, especially the F₁ strain, were hampered by small sample sizes. This was a result of very low feeding rates (approximately 10%) using the artificial membrane system. The low numbers of blood fed individuals limited our ability to estimate extrinsic incubation period (EIP) for *Ae. albopictus*, although based upon the complete infection and dissemination after 7 days at 28°C, we suspect the course of infection to be rapid.

The estimation of EIP for *Ae. aegypti* is important for parameterizing epidemiologic models and therefore predicting the speed and severity of an epidemic.³³ The F₁ *Ae. aegypti* had a higher percent disseminated and increased body titer over time, with decreasing differences in body titer between days 7 pe and 10 pe. Therefore, it is likely that under the conditions tested here, *Ae. aegypti* would be infectious within seven days of biting an infected human.

There were large statistical differences in dissemination (on all days) and body titer (on day 3 pe) between F₁ *Ae. aegypti* and Rockefeller *Ae. aegypti* demonstrates the importance of examining the course of infection at various times pe in different strains. This variation in the EIP between strains could impact epidemiologic risk.

The susceptibility of Florida mosquitoes of both species to CHIKV suggests the potential for emergence of this virus in Florida. However, other mitigating factors might limit this risk, specifically the behavior of Florida mosquitoes with regard to blood feeding, and the lower likelihood of human-vector contact in the United States, relative to less wealthy nations.³⁴ Furthermore, although *Ae. albopictus* was more susceptible in this study, the differences in ecology between these species, especially differences in their human biting rate, suggests more detailed information is needed before conclusions as to the vectorial capacity of each species can be made in a given location.

The spread of *Ae. albopictus* in the last 30 years¹⁹ and the importation of numerous, active cases of chikungunya fever to the United States^{9-11,35} emphasize the importance of knowing the relative vector competence of North American mosquitoes to this arbovirus. This knowledge, coupled with enhanced understanding of the ecology of the major vectors will enable locally specific, efficient deployment of public health resources in the event of an outbreak of CHIKV in North America.

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