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Abstract

Chikungunya virus is a re-emerging arbovirus of worldwide public health importance. This virus has been imported into the United States at an alarming rate via infected travelers. The primary vectors of this virus are *Aedes albopictus* and *Ae. aegypti* and *Culex pipiens quinquefasciatus* is one of the primary vectors transmitting other arboviruses to humans in Florida. It is widely known that different mosquito species and populations show varying levels of vector competence for pathogens. This report highlights the relationship between measures of vector competence and demonstrates that this virus could pose a public health disaster if it becomes established in Florida.

Introduction

Chikungunya virus (CHIKV, Family *Togaviridae*, genus *Alphavirus*) is a re-emerging arbovirus causing human epidemics in Europe, Africa, Asia, and India in recent years (Bessaud et al. 2006, Powers and Logue 2007, Panning et al. 2008, Peyrefitte 2008, Leo et al. 2009). The disease caused by CHIKV results in fever, skin rash, and arthritis-like pain in small peripheral joints that may last for weeks or months (Beltrame et al. 2007). There is currently no treatment for CHIKV infection and this is a serious public health hazard (Couderc et al. 2009). The primary human-mosquito-human transmission cycle of CHIKV increases the danger because international trade and travel can transport the virus to other locales, either within an infected human or mosquito. In 2007, a CHIKV outbreak causing illness in > 200 people in Italy was traced back to a single infected traveler who had recently returned from an area in India that was experiencing an epidemic (Rezza et al. 2007). The same Italian outbreak was attributed to international travel, exotic populations of *Ae. albopictus*, insufficient mosquito control, and environmental factors (Chretien and Linthicum 2007, Seyler et al. 2008). Similar records of imported cases of CHIKV have been reported in Singapore in 2007-2008 (Leo et al. 2009). There have been several reports of travelers inadvertently importing CHIKV into the United States (Beltrame et al. 2007) and Florida populations of both *Ae. albopictus* and *Ae. aegypti* are competent vectors of CHIKV based on reports of infection and dissemination rates (Reiskind et al. 2008, Pesko et al. 2009). *Aedes aegypti* and *Ae.*

albopictus are the primary vectors of CHIKV as they are particularly competent vectors both in the field and in the laboratory and feed primarily on humans (Bessaud et al. 2006, Powers and Logue 2007, Reiskind et al. 2008, Sang et al. 2008, Dubrulle et al. 2009). However, since *Culex pipiens quinquefasciatus* is an opportunistic feeder that also feeds on humans (Molaei et al. 2007), this species has the potential to be involved as a secondary vector in the CHIKV transmission cycle. Furthermore, an African field study reported CHIKV detected in strictly ornithophilic *Cx. ethiopicus*, indicating that birds may be one of the reservoir hosts during non-epidemic periods (Diallo et al. 1999, Chevillon et al. 2008) and lending more support for investigation of *Culex* spp. mosquitoes as vectors. Isolations of CHIKV have been found in field-collected *Cx. p. quinquefasciatus* during epidemics in Tanzania and Reunion Island (Ross 1956, Bessaud et al. 2006); however, published laboratory evaluations of vector competence of *Culex* spp. for CHIKV are lacking.

In this report, we evaluate vector competence measured as infection, dissemination, and transmission rates for an emergent strain of CHIKV in three Florida mosquito species that are known to blood feed on humans. This information will inform risk prediction models as mosquito control and public health agencies prepare for the possibility of a CHIKV outbreak in the United States, thereby targeting control measures to dangerous mosquito populations in advance of epidemics.

Methods

Mosquitoes. Three Florida mosquito colonies were used: *Ae. aegypti* colony established in 1952 from Alachua County in North Central Florida (generation > F_{50}), *Ae. albopictus* colony established in 1992 from Pinellas County in Western Florida (generation > F_{50}), and *Cx. p. quinquefasciatus* colony established in 2008 from Indian River County in Eastern Florida (generation = F_{25}). Mosquitoes were held in cages at 28˚C and 80% humidity at a 14:10 light:dark cycle and given 20% sugar and water *ad libitum*.

Virus propagation. Virus cultures were propagated according to standard procedures used in our laboratory and described elsewhere (Reiskind et al. 2008). African green monkey kidney (Vero) cells in T-75 cm^2 flasks are inoculated with 0.150 mL CHIKV (emergent Reunion strain LR2006-OPY1). After 48 h, supernatant was harvested and mixed with citrated bovine blood.

Mosquito infection. Vector competence assays were conducted using our previously established methods (Reiskind et al. 2008; Richards et al. 2007, 2009) and CHIKV was mixed with citrated bovine blood (Hemostat, Dixon, CA) prior to mosquito feeding. Samples (0.1 mL) of the blood meal were stored at -80^{o}C until further processing. Five day old mosquitoes were fed for 30 min on pledgets soaked with CHIKV-infected blood warmed (35˚C) for 10 min. Subsequent to feeding, mosquitoes were immobilized with cold and five freshly fed fully engorged mosquitoes from each treatment group frozen at -80^{o}C until further processing. The remaining fully engorged specimens were transferred to one-liter cardboard cages with mesh screening and maintained in incubators for 7 d at either 25^{o}C or 28^{o}C. Partially engorged or unfed mosquitoes were discarded. Mosquitoes were removed from cages, legs and wings detached and transferred to sample tubes containing 1.0 mL BA-1 diluent and saliva collected as previously described (Anderson et al. 2009; Richards et al. 2009). Mosquitoes were allowed to salivate for 45 min into capillary tubes filled with immersion oil. Bodies were also transferred to tubes containing 1.0 mL BA-1 diluent at the end of the salivation period. Bodies (without legs) were used to measure midgut infection and legs to measure virus dissemination out of the midgut. Virus in saliva was a measure of virus that had overcome salivary gland infection and escape barriers.

Virus assay. Two 4.5 mm zinc-plated beads were used to homogenize each body or leg sample at 25 Hz for 3 min (TissueLyser; Qiagen, Inc., Valencia, CA). Body, leg, and saliva samples were centrifuged at 4^{o}C and 3,148 x *g* for 4 min and viral RNA extracted using the MagNA Pure LC System and Total Nucleic acid Isolation Kit (Roche, Mannheim, Germany). The amount of viral RNA in each sample is determined using quantitative real-time Taqman reverse transcriptase polymerase chain reaction (qRT-PCR) (Lanciotti et al. 2000) with the LightCycler® 480 Instrument (Roche, Mannheim, Germany) and the Superscript III One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA) using a previously established program (Richards et al. 2007, Reiskind et al. 2008).

The infection rate is the percentage of all mosquitoes tested having infected bodies. The dissemination rate is the percentage of mosquitoes with infected bodies that also had infected legs. The transmission rate is the percentage of mosquitoes with infected bodies that also had infected saliva.

Results and Discussion

Mosquitoes were fed infectious blood meals containing 5.5 ± 0.1 (mean ± standard deviation) logs plaque-forming units (pfu) CHIKV/mL. Freshly fed mosquitoes (N=5 per species) collected immediately after blood feeding contained the following (logs pfu CHIKV/mL): *Ae. aegypti* (25°C: 4.1 ± 0.1; 28°C: 3.9 ± 0.2), *Ae. albopictus* (25°C: 3.8 ± 0.1; 28°C: 3.5 ± 0.2), and *Cx. p. quinquefasciatus* (25°C: 3.9 ± 0.2; 28°C: 4.1 ± 0.2). Results are listed in Table 1.

As very few *Cx. p. quinquefasciatus* in either of the two colonies tested became infected under the conditions of our test, these findings did not warrant development of the multiplex assay proposed in the initial proposal. However, we plan to use the information gained here as preliminary data and are developing ideas for identification of CHIKV-binding proteins (or lack thereof) in midgut tissues of these species and how this relates to vector competence. The finding that *Cx. p. quinquefasciatus* are incompetent vectors for CHIKV is important as we will use this information for comparison purposes. Table 1 shows that *Ae. albopictus* is a competent CHIKV vector and *Ae. aegypti* is a poor vector and the data gained from this study represents an important step in preparing Florida for a potential CHIKV outbreak. Further studies are needed to evaluate the extent to which additional virus doses and incubation temperatures would affect vector competence of Florida *Aedes* spp.

These data are currently being further analyzed statistically and results will be submitted for review to a peer-reviewed journal later this year, i.e. Richards, SL, Anderson, SL, and CT Smartt (2009) Vector competence of Florida *Culex* and *Aedes* (Diptera: Culicidae) for chikungunya virus. Results from these experiments will also be presented at the upcoming 2009 Annual Meeting of the Florida Mosquito Control Association in Tampa, FL.

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Table 1. The mean titers (logs plaque-forming units CHIKV/mL) ± standard deviation and rates of infection, dissemination, and transmission for *Aedes aegypti*, *Ae. albopictus*, and *Cx. p. quinquefasciatus* fed a CHIKV-infected blood meal with 5.5 ± 0.1 logs pfu/mL and held for 7 d at either 25°C or 28°C.

No. tested	**No. body infection (%)**	**No. leg infection (%)**	**No. saliva infection (%)**	**Body Titer**	**Leg Titer**	**Saliva Titer**
Aedes aegypti – 25°C						
48	5 (10)	3 (60)	0	3.7 ± 2.5	3.3 ± 2.2	-
Aedes albopictus– 25°C						
31	28 (90)	23 (82)	6 (21)	5.4 ± 0.8	4.5 ± 1.1	1.6 ± 0.7
Culex pipiens quinquefasciatus– 25°C						
50	0	0	0	-	-	-
Aedes aegypti - 28°C						
48	5 (10)	2 (40)	1 (20)	3.0 ± 2.4	4.8 ± 0.3	2.9
Aedes albopictus - 28°C						
30	29 (97)	21 (72)	2 (7)	5.3 ± 0.6	4.2 ± 1.2	1.9 ± 1.2
Culex pipiens quinquefasciatus– 28°C						
50	3 (6)	0	0	1.0 ± 0.3	-	-