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Susceptibility of a North American *Culex quinquefasciatus* to Japanese Encephalitis Virus

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Abstract

Japanese encephalitis virus (JEV) is a flavivirus that is transmitted by *Culex* (*Cx.*) *tritaeniorhynchus* in tropical and subtropical regions of Asia. The endemic transmission cycle involves domestic pigs and avian species that serve as amplification hosts; humans are incidental hosts that cannot develop a high-titer viremia sufficient for mosquito infection. Although vaccination can be an effective strategy for disease prevention and is used extensively in multiple Asian countries, unvaccinated immunologically naïve human populations can suffer from severe neurological sequelae. The potential introduction of JEV into North America would be a major threat to human and animal health. In this study, field-collected *Cx. quinquefasciatus* from Valdosta, Georgia, were tested for their susceptibility to JEV and their potential to develop a disseminated infection via *per os* infection. These results demonstrate that North American *Cx. quinquefasciatus* are susceptible to JEV infection and subsequent dissemination at 14 days post infection (d.p.i.). Detection of viral RNA in saliva from infected mosquitoes also indicates competent vectors for JEV can be found in North America.

Key Words: Japanese encephalitis virus—North American *Culex* species mosquitoes—Vector competence.

Introduction

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus primarily transmitted by *Culex* (*Cx.*) *tritaeniorhynchus* in Asia. Over the past 20 years, the distribution of JEV has undergone a significant expansion, spreading to new geographic areas such as Australia (van den Hurk et al. 2009). Although all known JEV cases identified in North America are associated with travel histories to endemic regions, the potential introduction of JEV remains a significant threat to human and veterinary public health in the United States due to the presence of large numbers of avian and swine species that could potentially be susceptible amplification hosts (Centers for Disease and Prevention 2005). As an exotic pathogen under the National Institute of Allergy and Infectious Diseases (NIAID) Category B Priority Pathogens for Biodefense Research, JEV can cause disastrous consequences if introduced into an immunologically naïve host population. Although JEV poses a potential threat, evaluation of the susceptibility and vector competence of North American mosquitoes for JEV has not been performed since 1946 (Reeves, et al. 1946). In this early study, colonized North American *Cx. quinquefasciatus* mosquitoes were infected orally from artificial blood meals containing the prototype strain Nakayama (genotype III); they transmitted the virus when allowed to probe on susceptible Swiss Webster strain laboratory mice.

The introduction of West Nile virus (WNV) in 1999 and chikungunya virus in 2013 has highlighted the possibility that exotic arboviruses can become established in the Americas when appropriate combinations of vectors and vertebrate hosts are present (Jia et al. 1999, Kuehn 2014). Therefore, it is critical to obtain information regarding the susceptibility of *Culex* mosquitoes that are present in the United States. Historically, genotype III isolates have caused numerous epidemics and have been characterized *in vitro* (Schuh et al. 2014). In this study, the susceptibility and likelihood of developing a disseminated form of infection among F_1 generation *Cx. quinquefasciatus* collected from Valdosta, Georgia, were tested by oral exposure of the JEV genotype III Taira

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strain. Transmission of JEV was further confirmed by the detection of viral nucleic acid in mosquito saliva.

Materials and Methods

Collection, rearing, and infection of mosquitoes

CDC gravid traps (CDC Gravid Trap Model 1712) baited with a hay infusion were used to collect F_0 generation *Cx. quinquefasciatus*. Adult mosquitoes were identified on the basis of morphological characteristics and maintained in 12-inch cages with 10% sucrose *ad libitum*. Blood meals and ovipositioning cups were provided to enable the production of eggs for the F_1 generation. Larvae hatched from egg rafts and pupae were maintained as previously described (Vanlandingham et al. 2004). Eight- to 10-day-old adults were challenged orally by JEV following deprivation of sugar for 48 h and water for 24 h. The JEV Taira strain was propagated in C6/36 cells at 28°C. Viremic blood meals were presented through Hemotek feeders and blood-soak cotton pledgets for 60 min followed by collection of engorged mosquitoes (Vanlandingham et al. 2004). Mosquitoes were maintained and dissected at 7 and 14 days post infection (d.p.i.) to obtain bodies and secondary tissues for the assessment of infection and dissemination rates. Virus-positive secondary tissues (head, legs, and wings) of individual mosquitoes are indicative of a disseminated infection. Whole mosquitoes were also collected to characterize viral replication at the same time points.

Detection of JEV in mosquito tissues and saliva

All samples were homogenized and titrated using a 50% tissue culture infectious dose ($TCID_{50}$) method in Vero76 cells, as previously described (Vanlandingham et al. 2004). The infection rate was determined by the percentage of homogenized mosquito tissues or carcasses that demonstrated virus-induced cytopathic effect (CPE) divided by the numbers of mosquitoes tested. The dissemination rate was calculated based on the percentage of homogenized secondary tissues that caused CPE divided by the number of mosquitoes dissected and tested positive.

To assess transmission capacity, 14 d.p.i. saliva from individual mosquitoes was collected by placing the proboscis of anesthetized mosquitoes into capillary tubes filled with mineral oil (Vanlandingham et al. 2004). Viral RNA was extracted with a QIAamp Viral RNA Kit (Qiagen, Valencia, CA), reverse transcribed with Superscript III reverse transcriptase (Life Technologies, Carlsbad, CA), and amplified by seminested PCR with JEV-specific primers (Johansen et al. 2002).

Results and Discussion

Engorgement with JEV presented in artificial blood meals resulted in an average titer of 4.8 $logTCID_{50}$/mL ($n = 3$) among mosquitoes collected immediately following *per os* infection. The infection and dissemination rates at 7 and 14 d.p.i. are summarized in Table 1. At 7 d.p.i., the infection rate was 100.0% (12/12); however, none of the infected mosquitoes demonstrated evidence of a disseminated infection. At 14 d.p.i., the infection rate was 84.6% (22/26). Among 18 dissected mosquitoes, 14 (77.8%) were positive for JEV in the homogenized body segment, and 50.0% (7/14) developed disseminated infection. The average whole-body mosquito JEV titers were 4.2 $logTCID_{50}$/mL and 4.5 $logTCID_{50}$/mL at 7 and 14 d.p.i., respectively. The detection of viral RNA in the saliva identified two JEV-positive saliva samples among 22 infected mosquitoes leading to a 9.1% (2/22) transmission rate.

Table 1. Infection Rate, Dissemination Rate, and Average Whole-Mosquito Titer of Japanese Encepahlitis Virus at 7 and 14 Days Postinfection

	7 d.p.i.	*14 d.p.i.*
Infection rate	100.0% (12/12)	84.6% (22/26)
Dissemination rate	0.0% (0/8)	50.0% (7/14)
Whole-mosquito titer ($logTCID_{50}$/mL)	4.2	4.5

d.p.i., days postinfection; $TCID_{50}$, 50% tissue culture infectious dose.

Our results demonstrate that F_1 generation *Cx. quinquefasciatus* collected from Valdosta, Georgia, are susceptible to JEV infection. Subsequent transmission was also observed, albeit at a relatively low rate. This is the first evidence demonstrating field-collected *Cx. quinquefasciatus* in North America can be orally infected by JEV. Disseminated infections observed at 14 d.p.i. indicate that JEV replicated and escaped from the infected midgut into secondary tissues. Dissemination of virus to secondary tissues further led to the transmission through the secretion of saliva. Dissemination was only observed at 14 d.p.i. and is consistent with a previous observation that JEV requires a 6- to 10-day-long extrinsic incubation period in *Cx. fuscocephala* to initiate the transmission (Muangman et al. 1972). Whereas the results presented in previous studies have clearly shown that colonized North American *Culex* mosquitoes are susceptible to JEV and able to transmit the virus (Reeves et al. 1946), our study has filled a significant gap in the epidemiological data by showing that field-collected North American mosquitoes are susceptible to JEV infection. Despite genetic differences between the prototype Nakayama strain and the more recent isolate Taira strain and different methods of virus propagation, these two studies performed over 60 years apart demonstrated transmission of JEV by North American mosquitoes.

A biological transmission cycle requires the presence of susceptible vertebrate species capable of developing sufficiently high viremic titers. Previously, several avian species in North America have been demonstrated to be susceptible to JEV and can potentially serve as amplification hosts (Nemeth et al. 2012). Therefore, it is important to investigate further whether domestic or feral swine populations from North America are susceptible to JEV and could subsequently serve as amplifying hosts. Such knowledge is critical to assess the potential for JEV to establish local transmission cycles similar to WNV in North America.

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Author Disclosure Statement

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