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Research article

Potential of a Northern Population of *Aedes vexans* (Diptera: Culicidae) to Transmit Zika Virus

Kyle L. O'Donnell,[1] Mckenzie A. Bixby,[2] Kelsey J. Morin,[2] David S. Bradley,[1] and Jefferson A. Vaughan[2,3]

[1]Department of Basic Sciences, University of North Dakota School of Medicine and Health Sciences, Grand Forks, ND 58202 (kyle.odonnell@und.edu; david.s.bradley@med.und.edu), [2]Department of Biology, University of North Dakota, Grand Forks, ND 58202 (mckenzie.bixby@und.edu; kelsey.j.morin@und.edu; jefferson.vaughan@email.und.edu), and [3]Corresponding author, e-mail: jefferson.vaughan@email.und.edu

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Abstract

Zika virus is an emerging arbovirus of humans in the western hemisphere. With its potential spread into new geographical areas, it is important to define the vector competence of native mosquito species. We tested the vector competency of *Aedes vexans* (Meigen) from the Lake Agassiz Plain of northwestern Minnesota and northeastern North Dakota. *Aedes aegypti* (L.) was used as a positive control for comparison. Mosquitoes were fed blood containing Zika virus and 2 wk later were tested for viral infection and dissemination. *Aedes vexans* ($n = 60$) were susceptible to midgut infection (28% infection rate) but displayed a fairly restrictive midgut escape barrier (3% dissemination rate). Cofed *Ae. aegypti* ($n = 22$) displayed significantly higher rates of midgut infection (61%) and dissemination (22%). To test virus transmission, mosquitoes were inoculated with virus and 16–17 d later, tested for their ability to transmit virus into fluid-filled capillary tubes. Unexpectedly, the transmission rate was significantly higher for *Ae. vexans* (34%, $n = 47$) than for *Ae. aegypti* (5%, $n = 22$). The overall transmission potential for *Ae. vexans* to transmit Zika virus was 1%. Because of its wide geographic distribution, often extreme abundance, and aggressive human biting activity, *Ae. vexans* could serve as a potential vector for Zika virus in northern latitudes where the conventional vectors, *Ae. aegypti* and *Ae. albopictus* Skuse, cannot survive. However, Zika virus is a primate virus and humans are the only amplifying host species in northern latitudes. To serve as a vector of Zika virus, *Ae. vexans* must feed repeatedly on humans. Defining the propensity of *Ae. vexans* to feed repeatedly on humans will be key to understanding its role as a potential vector of Zika virus.

Key words: *Aedes vexans*, arbovirus, mosquito, Zika virus

Zika virus (family *Flaviviridae*) is a mosquito-borne virus of primates in sub-Saharan Africa that has spread rapidly within the past decade to cause serious epidemics throughout the western Pacific and Latin America (Musso and Gubler 2016). Several features of Zika virus make its spread particularly troubling. In addition to being transmitted by infective mosquitoes, Zika virus can also be transmitted sexually (Moreira et al. 2017). No other arbovirus is known to be sexually transmitted. In most cases, Zika virus infections in humans do not produce life-threatening illness, but in some instances, Zika virus infections can lead to a neuropathic condition known as Guillain-Barré syndrome, and in pregnant women, Zika viral infection can infect the fetus causing a brain abnormality known as microcephaly in the unborn child (Krauer et al. 2017). Sylvatic circulation of Zika virus in Africa involves primarily monkeys and several species of tree hole and container-breeding *Aedes* spp., including *Aedes africanus* (Theobold) (Dick et al. 1952), *Aedes luteocephalus* (Newstead), and *Aedes vittatus* Bigot (Diagne et al. 2015). Urban circulation involves a human–mosquito–human cycle with *Aedes aegypti* (L.) as the primary vector (Li et al. 2012). Other mosquito species have been implicated as competent vectors of Zika virus, most notably *Aedes albopictus* (Skuse) (Wong et al. 2013, Di Luca et al. 2016), and to a lesser extent, *Aedes hensilli* Farner (Ledermann et al. 2014), and some (Guo et al. 2016) but not all strains within the *Culex pipiens* L./*Culex quinquefasciatus* Say complex (Aliota et al. 2016a, Huang et al. 2016, Fernandes et al. 2016, Boccolini et al. 2016, Hall-Mendelin et al. 2016, Hart et al. 2017). In this report, we tested *Aedes vexans* (Meigen) from the upper Great Plains for their ability to transmit Zika virus.

Materials and Methods

Mosquitoes

Host-seeking mosquitoes were collected using a Mosquito Magnet X trap baited with bottled CO_2 released at a flow rate of ca. 500 ml

per minute. The trap was operated overnight at rural residences in Polk County, MN (31 July 2016), and Grand Forks County, ND (22 September 2016). Both sites are located within the Lake Agassiz Plain eco-region of northwestern Minnesota and northeastern North Dakota. The next morning, the trap was transported to the laboratory and mosquitoes were released into a large, cubic-meter screened cage. The most abundant species (ca. 85% collected) was the inland floodwater mosquito, *Ae. vexans*. Mosquitoes were maintained for several days on cotton pads soaked in 10% sugar water and 0.05% antibiotic solution. Antibiotics were given to eliminate variation in bacterial loads within the alimentary tracts of wild-caught mosquitoes and thus minimize any potential confounding effects that midgut bacteria may exert on the infectivity of experimentally administered virus (Ramirez et al. 2012). As a positive control for vector competence, an *Ae. aegypti* colony (Costa Rica strain, F39) was established from eggs obtained from BEI Resources. Female *Ae. aegypti* mosquitoes were likewise maintained for several days on the glucose–antibiotic solution prior to testing.

Virus

The strain of Zika virus used in this study was originally isolated from a patient in Puerto Rico in 2016. Viral stocks were prepared after two passages of the isolation onto Vero cells maintained at 37 °C. Viral titer was estimated via a plaque assay. Serial 10-fold dilutions were inoculated onto Vero cells overlaid with 1% methylcellulose, which were then incubated for 7 d. The overlay was then removed and the monolayer was fixed with 10% formaldehyde and stained with crystal violet. Plaques were counted and the resulting titer was expressed as plaque-forming units per milliliter (PFU/ml). The viral stock was diluted 1:1 with heat inactivated fetal bovine serum then divided into 1-ml aliquots and stored at −80 °C.

Oral Exposure of Mosquitoes

Prior to exposure, mosquitoes were transferred to 3.8-liter cylindrical cardboard cages (ca. 50 per cage) each fitted with a screened top secured with tape and a single high-security double-layered dental dam access portal. Infectious blood consisted of Zika virus culture media mixed 1:1 with de-fibrinated cow blood (Pel-Freez Biologicals, Rodgers, AK). The first trial (Minnesota mosquitoes plus *Ae. aegypti*) used thawed virus fed to mosquitoes at an estimated bloodmeal concentration 9.2×10^6 plaque-forming units per milliliter (PFU/ml). The second trial (North Dakota *Ae. vexans*) used fresh virus grown on Vero cells prior to mosquito feeding and with an estimated bloodmeal concentration of 2.0×10^5 PFU/ml. Bloodmeals were administered via water-jacketed membrane feeders (circulating water at ca. 38 °C) fitted with de-salted pork sausage casing. Mosquitoes were given 1 h to feed, after which unfed mosquitoes were removed. Cages containing engorged mosquitoes were placed within transparent plastic tubs and maintained on glucose–antibiotic solution for 14 d in a biosafety level-2 insectary with restricted access and environmental settings at 28 °C and a photoperiod of 16:8 (L:D) h. At 14 d, mosquitoes were killed by freezing at −20 °C for ≥ 2 h. For each mosquito, the legs were pulled off and the bodies (=infection) and mosquito legs (=disseminated infection) were ground separately in 200 µl grinding solution (M-199 + 5% calf serum + 0.5% antibiotics).

Parenteral Infection and Salivary Transmission

An aliquot of the same virus batch used in our first feeding trial was thawed and inoculated into *Ae. aegypti* and *Ae. vexans* mosquitoes to compare their abilities to transmit virus orally. Mosquitoes were immobilized by chilling (1 min in −20 °C freezer) in batches of 5–15 each and placed on a chill table (BioQuip, Rancho Dominguez, CA). Mosquitoes were injected intrathoracically with 0.3 µl of media containing 1.8×10^7 PFU/ml using a glass needle powered by a microinjection pump (TriTech Research Inc., Los Angeles, CA). Following injection, mosquitoes were placed into 0.5-liter cylindrical cardboard cages and maintained as described above. On 16–17 d after injection, mosquitoes were tested for their ability to secrete virus in their saliva (Anderson et al. 2010). To do this, three to five mosquitoes at a time were chilled, legs amputated and then placed on a strip of double-stick tape running along the edge of a glass plate. Mosquito were carefully positioned on the plate so that their proboscises were free and hanging over the edge. Immediately after mosquitoes were in position, a small amount of malathion insecticide (0.4 µl of 0.14% AI in acetone) was applied to the thorax of each mosquito to stimulate salivation (Boorman 1987). After 10–15 min, this plate was abutted against a second glass plate to which capillary tubes containing ca. 20 µl of M-199 media plus 10% calf serum were affixed. Each mosquito proboscis was carefully inserted into a liquid-filled capillary tube and mosquitoes were given 20–30 min to salivate. To determine whether or not mosquitoes were actually salivating and thus potentially transmitting virus, mosquitoes were examined every 2–3 min throughout the trial under a stereoscope for secretion of clear saliva into the pinkish media or ingestion of fluid (expanded crop). After the allotted time, capillary tubes were collected and the contents expelled into individual microfuge tubes containing 50 µl of media. Mosquitoes were also placed into individual microfuge tubes to test for disseminated viral infection. All microfuge tubes were labeled so that expectorate samples could be matched to the individual mosquitoes from which they had been collected. Microfuge tubes were stored at −20°C until processed for Zika viral RNA detection. Expectorate samples were tested for virus from all body-positive mosquitoes whether or not mosquitoes had been observed to salivate or imbibe fluid from capillary tubes.

Zika Viral RNA Detection

Viral RNA was detected using reverse transcriptase polymerase chain reaction (RT-PCR) techniques. Frozen triturates of mosquito bodies and legs were thawed, centrifuged at 14,500 rpm for 5 min, and 140 µl of supernatant was extracted for RNA using Qiagen QIAamp Viral RNA Mini Kits according to manufacturer's instructions. Real time PCR was conducted using Qiagen one-step RT-PCR kit with primers specific for the envelope gene. Probe sequence: 5′-56FAM/ACGCCTAAT/ZEN/TCACCAAGAGCGGAA/3IABkFQ-3′, Primer 1: 5′-TCCTAAGCTTCCAAAGCCTCCCAA-3′, and Primer 2: 5′-TATCAGTGCATGGCTCCCAGCATA-3′. Cycle parameters were 1) 30 min at 50 °C, 2) 15 min at 95 °C, 3) 40 cycles of—1 min at 94 °C, 1 min at 54 °C, 1 min at 72 °C, and 4) 10 min 72 °C. Reactions were performed using CFX96 IVD Real-Time PCR Systems and accompanying software to determine cut-off values based on at least two negative controls (water only) and two positive controls (Zika viral culture extracts) per assay.

Data Analysis

The infection rate was the percentage of orally exposed mosquitoes tested that contained viral RNA 14 d after feeding on viremic blood. The dissemination rate was the percentage of orally exposed mosquitoes tested that contained viral RNA in their legs (regardless of their infection status) 14 d after feeding on viremic blood. The transmission rate was the percentage of virus-inoculated mosquitoes with

Table 1. Rates of Zika virus infection and dissemination (number tested, 95% confidence interval) in mosquitoes 14 d after ingesting defibrinated blood containing virus

Mosquito species	Mosquito source	Virus source	Virus concn (PFU/ml)	% Infection[a]	% Dissemination[b]	% Dissemination/ Infection[c]
Aedes aegypti	Colonized; Costa Rica strain (BEI Resources)	Thawed	9.2×10^6	61 (18, 36–83)	22 (18, 6–48)	36 (11, 11–69)
Aedes vexans	Wild-caught; Polk Co., MN	Thawed	9.2×10^6	29 (28, 13–49)	4 (28, <1–18)	12 (8, <1–53)
	Wild-caught; Grand Forks Co., ND	Fresh	2.0×10^5	28 (32, 14–47)	3 (32, <1–16)	11 (9, <1–48)

[a] Percentage of mosquitoes containing virus in their bodies (number positive/number tested).
[b] Percentage of mosquitoes containing virus in their legs (number positive/number tested).
[c] Percentage of infected mosquitoes containing virus in their legs (number positive/number tested).

disseminated viral infections that transmitted virus into fluid-filled capillary tubes 16–17 d after inoculation. Rates of viral infection, dissemination, transmission, and fluid ingestion or salivation were compared among groups by chi-square analyses or Fisher's exact test, depending on sample size, using the software package, Statistix (Tallahassee, FL) with the 0.05 level of significance throughout. The exact (bionomial) method was used to calculate 95% confidence intervals (https://measuringu.com/wald/, accessed on 11 March 2017).

Results

Susceptibility to Oral Infection and Viral Dissemination

Membrane feeding success was higher for colonized *Ae. aegypti* (69%, $n = 39$) than for wild-caught *Ae. vexans* (43%, $n = 168$; $\chi^2 = 8.82$, $P = 0.003$). *Aedes aegypti* had significantly higher rates of viral infection and dissemination than *Ae. vexans* (Fisher exact tests, $P < 0.024$). Seventeen of 60 *Ae. vexans* tested (28%) became infected and two (3%) developed disseminated infections, indicating a moderately severe midgut escape barrier for Zika virus in this species. Eleven of 18 *Ae. aegypti* tested (61%) became infected and four (22%) developed disseminated infections (Table 1). However, the proportion of infected mosquitoes having disseminated infections did not differ statistically between *Ae. aegypti* (4 of 11) and *Ae. vexans* (2 of 17; Table 1; Fisher exact test, $P = 0.14$). Interestingly, there were no differences in infection or dissemination rates between *Ae. vexans* fed fresh versus thawed virus (P's > 0.59, Fisher exact tests), even though thawed virus had nearly 100-fold higher titer (Table 1). This supports earlier findings that freshly cultured Zika virus is more infective to mosquitoes than frozen virus that is thawed just prior to feeding to mosquitoes (Weger-Lucarelli et al. 2016).

Virus Transmission by Inoculated Mosquitoes

All 47 inoculated *Ae. vexans* and 22 of 23 inoculated *Ae. aegypti* were positive for Zika virus. The virus transmission rates for *Ae. vexans* ($n = 47$) and *Ae. aegypti* ($n = 22$) were 34% and 5%, respectively (Table 2; Fisher exact test, $P = 0.06$). Whether or not a mosquito was observed to salivate into or imbibe fluid from its capillary tubes had no bearing on whether or not it actually transmitted virus. For example, the transmission rate for the 33 *Ae. vexans* observed to either salivate or imbibe fluid (36%) did not differ significantly from the transmission rate for the 14 *Ae. vexans* not observed to salivate or imbibe (40%; Fisher exact test, $P = 0.43$). The single *Ae. aegypti* that transmitted virus was not observed to salivate nor imbibe fluid. Transmission potentials, calculated as (dissemination rate of all mosquitoes tested, Table 1) × (transmission rate of mosquitoes with disseminated infections, Table 2), were virtually identical between *Ae. aegypti* (1.0%) and *Ae. vexans* (1.1%).

Table 2. Rates of Zika virus transmission (number tested, 95% confidence interval) by mosquitoes 16–17 d after being inoculated intrathoracically with 0.3 µl of media containing 1.8×10^7 plaque-forming units per milliliter of virus

Mosquito species	Mosquito origin	% Transmission[a]
Aedes aegypti	Colonized; Costa Rica strain (BEI Resources)	5 (22, <1–23)
Aedes vexans	Wild-caught; Grand Forks Co., ND	34 (47, 21–49)

[a] Percentage of mosquitoes with a disseminated viral infection that expectorated virus into fluid-filled capillary tubes following a topical application of 0.4 µl acetone containing malathion (0.14% AI) to induce salivation.

Discussion

This study indicates that *Ae. vexans* from the upper Great Plains is physiologically capable of becoming orally infected and transmitting Zika virus. *Aedes vexans* displayed moderate to severe midgut and midgut escape barriers to virus infection and dissemination, respectively. However, once disseminated infections were established, the transmission rate of Zika virus by *Ae. vexans* (34%, $n = 47$) was comparable to transmission rates reported at similar incubation periods (i.e., ≥ 14 d) for some (but not all) strain combinations of Zika virus and *Ae. aegypti* (i.e., Aliota et al. 2016b [33%, $n = 12$], Chouin-Carneiro et al. 2016 [21%, $n = 14$], Richard et al. 2016 [42%, $n = 33$], Costa-da-Silva et al. 2017 [5%, $n = 189$], Heitmann et al. 2017 [31%, $n = 36$]).

Aedes vexans is the first indigenous North American mosquito species found capable of transmitting Zika virus under laboratory conditions. Several other mosquito species have been shown in laboratory studies to be refractory to Zika virus, including *Aedes triseriatus* (Say) (Aliota et al. 2016b), *Aedes taeniorhynchus* (Wiedemann) (Hart et al. 2017), and *Culex tarsalis* (Coq.) (Weger-Lucarelli et al. 2016) from North America, *Aedes polynesiensis* Marks from French Polynesia (Richard et al. 2016), and *Aedes notoscriptus* (Skuse), *Aedes procax* (Skuse), *Aedes vigilax* (Skuse), *Culex annulirostris* Skuse, and *Culex sitiens* Wiedemann from Australia (Hall-Mendelin et al. 2016).

The primary urban vectors of Zika are *Ae. aegypti* and *Ae. albopictus*. As Zika virus spread from Africa into Asia, Oceania, and the Western Hemisphere, the virus encountered novel populations and strains of these vector species. It is now clear from recent vector competency studies that the efficiencies by which *Ae. aegypti* and *Ae. albopictus* transmit Zika virus varies greatly depending on the strains of mosquito and virus examined. In our study, the rates of viral infection (61%) and dissemination (22%) for Zika virus in our

Ae. aegypti strain were comparable to other strain combinations examined, yet the transmission rate (5%, Table 2) was uncharacteristically low. We had purposely selected our "positive control system"—i.e., a Latin American strain of *Ae. aegypti* (Costa Rica) and a Caribbean isolate of Zika virus (Puerto Rico)—based on the assumption that the close geographic proximity of origin between mosquito strain and virus isolate would result in optimal virus transmission. This proved not to be the case. But this is not the first example of incompatibility between geographically proximate strains. Interestingly, two separate strains of *Ae. aegypti* from Senegal have been reported to have severely limiting midgut escape barriers and insurmountable salivary gland barriers to the native Sengalese strain of Zika virus (Diagne et al. 2015). Thus, *Ae. aegypti* is probably not the vector for Zika virus in Senegal. Conversely, a strain of *Ae. aegypti* from Mexico produced a significantly higher rate of disseminated infection with a Zika virus strain from Senegal (60%, $n = 48$) than with Zika virus isolated from a patient in Puerto Rico (42%, $n = 48$; Weger-Lucarelli et al. 2016). These studies indicate that the combination of mosquito and virus strains in the *Ae. aegypti*–Zika system greatly influences vector competency although the outcome cannot be predicted based on geographic origins of strains and isolates. *Aedes aegypti* is widely distributed throughout tropical latitudes and analogously, *Ae. vexans* is widely distributed throughout temperate and subarctic latitudes. Thus, it may be anticipated that *Ae. vexans* may also display geographic heterogeneity in vector competence to Zika virus. Such heterogeneity has been shown to occur with *Ae. vexans* and Rift Valley fever virus, where southern populations of North American *Ae. vexans* are susceptible but northern populations are not (Turell et al. 2008, 2010; Iranpour et al. 2011; Turell et al. 2013).

Beyond its ability to transmit the virus, there are other characteristics of *Ae. vexans* that could contribute to its role as a potential vector of Zika virus in the Northern Hemisphere. First, *Ae. vexans* is an aggressive, nearly cosmopolitan Holarctic mosquito species with a long flight range (Clarke 1943, Brust 1980, Briegel et al. 2001, Szalanski et al. 2006, Francuski et al. 2016). It feeds primarily on large mammals and readily attacks humans during both day and night (Magnarelli 1977, Burkot and DeFoliart 1982, Vaughan et al. 2012, Greenberg et al. 2013, Mehus and Vaughan 2013). Second, *Ae. vexans* is extremely prolific and often cited as the most abundant species found in mosquito surveys conducted throughout the northern hemisphere, including central Canada (Henderson et al. 2006, Schofield et al. 2007), most of the United States (Easton et al. 1986, Rooker et al. 1994, Russo 1997, Meece et al. 2003, Andreadis et al. 2004, Bell et al. 2005, Bolling et al. 2005, Eisen et al. 2008, McPhatter et al. 2012, Anderson et al. 2015), France (Balenghien et al. 2006), Germany (Lühken et al. 2014), Czech Republic (Berec et al. 2014), Slovakia (Bocková et al. 2015), Hungary (Kemenesi et al. 2015), Croatia (Merdić and Lovaković. 2001, Merdić et al. 2014), Russia (Fyodorova et al. 2006), Iran (Yaghoobi-Ershadi et al. 2016), South Korea (Burkett et al. 2002), and China (Wang et al. 2012). Third, *Ae. vexans* has been shown in laboratory studies to be a competent vector for several arboviruses, including West Nile virus (Goddard et al. 2002, Tiawsirisup et al. 2008), eastern equine encephalomyelitis virus (Vaidyanathan et al. 1997), Saint Louis encephalitis virus (Hammon and Reeves 1943), Rift Valley fever virus (Ndiaye et al. 2016, Turell et al. 2010), Tahyna virus (Rödl et al. 1979), and Geta virus (Takashima et al. 1983). Thus, it would seem that *Ae. vexans* could potentially play a role in transmitting Zika virus if the virus were to be introduced into more northerly latitudes of North America or Eurasia where the primary vectors, *Ae. aegypti* and *Ae. albopictus*, are absent.

On the other hand, Zika virus is a virus of primates. Outside the tropics, humans are essentially the only amplifying host. Thus, for *Ae. vexans* to be a vector of Zika virus in northern latitudes, it would have to feed on a viremic human, survive the extrinsic incubation period of the virus, and then feed again on a human. Using mark–release–recapture techniques, Jensen and Washino (1994) estimated the field survivorship of *Ae. vexans* in California to be fairly low (daily survival = 0.70) compared to typical values reported for *Ae. aegypti* (e.g., 0.80 to 0.95). Low field survival would reduce the overall vectorial capacity of *Ae. vexans*. Nevertheless, densities of host-seeking *Ae. vexans* can be extreme, even in residential areas (Bell et al. 2005). Prodigious numbers may compensate for low daily survival and weak vector competence, and thus elevate the importance of *Ae. vexans* as a vector for zoonotic arboviruses in some regions (see Anderson et al. 2015). But again, the transmission pattern of Zika virus in the north would undoubtedly be human-to-mosquito-to-human. Thus, information regarding the propensity of *Ae. vexans* to feed repeatedly on humans is key in evaluating its potential as a vector of Zika virus. In the rural Midwest, *Ae. vexans* feed primarily on deer (Burkot and DeFoliart 1982, Mehus and Vaughan 2013). But the primary bloodmeal sources for *Ae. vexans* are less well-defined in suburban and urban areas where human density, and hence human availability as blood sources, typically exceeds that of deer and other large mammals. To help define the actual vector potential of *Ae. vexans* for Zika virus in North America, mosquito surveillance programs could include pools of field-collected *Ae. vexans* as part of their Zika virus testing—particularly in the southeast USA where *Ae. vexans* and *Ae. aegypti* co-occur and the threat of local virus amplification exists. This may reveal whether or not *Ae. vexans* mosquitoes are being naturally exposed to Zika virus.

Additional vector competence studies with *Ae. vexans* (e.g., comparing geographic strains, defining extrinsic incubation period, transovarial transmission, etc.) are warranted because of this species' expansive distribution, considerable dispersal capability, often excessive abundance, and propensity to attack humans. Other Holarctic anthropophagic mosquito species should also be examined.

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