Differential Vector Competency of *Aedes albopictus* Populations from the Americas for Zika Virus

Sasha R. Azar,[1,2,3]† Christopher M. Roundy,[1,2,3]† Shannan L. Rossi,[1,2] Jing H. Huang,[1,2] Grace Leal,[1,2]
Ruimei Yun,[1,2] Ildefonso Fernandez-Salas,[4] Christopher J. Vitek,[5] Igor A. D. Paploski,[6,7] Pamela M. Stark,[8]
Jeremy Vela,[8] Mustapha Debboun,[8] Martin Reyna,[8] Uriel Kitron,[9] Guilherme S. Ribeiro,[6,7]
Kathryn A. Hanley,[10] Nikos Vasilakis,[1,2]*† and Scott C. Weaver[1,2,3]*†

[1]Department of Pathology, Institute for Human Infections and Immunity, University of Texas Medical Branch, Galveston, Texas; [2]Center for Tropical Diseases, University of Texas Medical Branch, Galveston, Texas; [3]Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas; [4]Instituto Nacional de Salud Pública, Centro Regional de Salud Pública, Tapachula, Chiapas, México; [5]University of Texas Rio Grande Valley, Edinburg, Texas; [6]Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Ministério da Saúde, Candeal, Salvador, Brazil; [7]Instituto de Saúde Coletiva, Universidade Federal da Bahia, Salvador, Brazil; [8]Mosquito and Vector Control Division, Harris County Public Health, Houston, Texas; [9]Population Biology, Ecology, and Evolution Graduate Program, Graduate Division of Biological and Biomedical Sciences, Department of Environmental Sciences, Emory University, Atlanta, Georgia; [10]Department of Biology, New Mexico State University, Las Cruces, New Mexico

Abstract. To evaluate the potential role of *Aedes albopictu*s (Skuse) as a vector of Zika virus (ZIKV), colonized mosquitoes of low generation number (≤ F5) from Brazil, Houston, and the Rio Grande Valley of Texas engorged on viremic mice infected with ZIKV strains originating from Senegal, Cambodia, Mexico, Brazil, or Puerto Rico. Vector competence was established by monitoring infection, dissemination, and transmission potential after 3, 7, and 14 days of extrinsic incubation. Positive saliva samples were assayed for infectious titer. Although all three mosquito populations were susceptible to all ZIKV strains, rates of infection, dissemination, and transmission differed among mosquito and virus strains. *Aedes albopictus* from Salvador, Brazil, were the least efficient vectors, demonstrating susceptibility to infection to two American strains of ZIKV but failing to shed virus in saliva. Mosquitoes from the Rio Grande Valley were the most efficient vectors and were capable of shedding all three tested ZIKV strains into saliva after 14 days of extrinsic incubation. In particular, ZIKV strain DakAR 41525 (Senegal 1954) was significantly more efficient at dissemination and saliva deposition than the others tested in Rio Grande mosquitoes. Overall, our data indicate that, while *Ae. albopictus* is capable of transmitting ZIKV, its competence is potentially dependent on geographic origin of both the mosquito population and the viral strain.

INTRODUCTION

Zika virus (ZIKV) is an emerging mosquito-borne flavivirus in the family *Flaviviridae,* which includes human pathogens such as yellow fever, dengue viruses (DENVs), and West Nile virus (WNV). ZIKV was originally isolated from the blood of a sentinel rhesus macaque placed in a canopy platform in the Zika forest of Uganda in 1947.[1] The virus circulates in several regions of Africa between arboreal mosquitoes such as *Aedes africanus* (Theobald) and *Aedes furcifer* (Edwards) and nonhuman primates in what is called an enzootic or sylvatic cycle.[2] However, "spillover" infections in Africa are rarely detected, with only 14 human cases reported in the medical literature until contemporary outbreaks beginning in 2007.[3] In 2007, independent ZIKV epidemics occurred in Gabon[4] and Yap Island in the Federated States of Micronesia,[5] the latter estimated to have affected 73% of the island's population of 7,391 persons. Following the path of another arthropod-borne virus (arbovirus), chikungunya virus (CHIKV), Zika began spreading in the South Pacific in 2013, affecting several islands, including French Polynesia, New Caledonia, Easter Island, and the Cook Islands.[6–8] In early 2015, transmission was first detected in northeastern Brazil,[9,10] followed by explosive spread throughout the Americas, with transmission reported in 48 countries and territories, including Florida and Texas in the United States.[11,12]

Infection with ZIKV is inapparent or subclinical in a majority (up to 80%) of people[13] and symptomatic cases are generally characterized by mild signs and symptoms such as fever, headache, malaise, conjunctivitis, myalgia, arthralgia, and maculopapular rash.[13–15] This syndrome can closely resemble that caused by other arboviruses such as CHIKV and DENV, which co-circulate with ZIKV in many regions. However, a spike in reports of serious outcomes of ZIKV infection such as microcephaly,[13,16] neurological, ocular and muscular complications,[17–19] and Guillain–Barré syndrome that have been identified in the Americas, and retrospectively during in the French Polynesian outbreak,[20,21] caused the World Health Organization to declare ZIKV an international public health concern.[22]

In the absence of therapeutics or vaccines, control of ZIKV outbreaks is limited to mosquito abatement and prevention of mosquito–human contact. Since its discovery in 1947, ZIKV has been isolated from several species of mosquitoes within the genus *Aedes*, including several arboreal species such as *Ae. furcifer*, *Ae. luteocephalus* (Newsteed), and *Ae. africanus*.[1,23,24] ZIKV was first isolated from a peridomestic mosquito, *Ae. aegypti* (Linnaeus) in 1969 in Malaysia,[25] and later implicated in outbreaks in the Pacific due to the presence of ZIKV RNA in a single *Ae. aegypti* pool.[26] However, the role of *Ae. aegypti* in outbreaks was not directly confirmed until this species was implicated in a 2015 outbreak of ZIKV in Chiapas State, Mexico,[27] and later in Rio de Janeiro, Brazil.[28] Other vectors have also been evaluated for their role in transmission; for example, *Ae.* (*Stegomyia*) *hensilli* Farner was retrospectively identified as the likely vector on Yap Island in 2007.[3]

* Address correspondence to Nikos Vasilakis or Scott C. Weaver, University of Texas Medical Branch, 301 University Blvd., Galveston TX 77555-0609. E-mails: nivasila@utmb.edu or sweaver@utmb.edu
† These authors contributed equally to this work.

Another mosquito suspected of urban ZIKV transmission is *Ae. albopictus*, the Asian tiger mosquito. Originating in the forests of southeast Asia, this invasive species recently spread throughout tropical and temperate regions of Asia, Europe, the Americas, and Africa.[29] When compared with its close relative *Ae. aegypti*, *Ae. albopictus* is regarded as a secondary vector for several arboviruses, including DENV. In recent years, this prevailing view has changed, primarily due to the role of *Ae. albopictus* in Indian Ocean Basin and Asian CHIKV outbreaks. This role was facilitated by the convergent evolution in these regions of substitutions at position 226 of the envelope glycoprotein one involving Ala → Val (E1-A226V), which confers an approximately 40-fold fitness gain for transmission by *Ae. albopictus*, followed by additional adaptive substitutions in the E2 protein in Asia.[30–32] In locations where *Ae. albopictus* is the predominant (or only) vector of CHIKV, such as in La Réunion, Italy, Cameroon, France, Gabon, and parts of Thailand, these *Ae. albopictus*-adapted strains have displaced others.[31]

Due to its ecological plasticity, which in part has facilitated its invasive spread, *Ae. albopictus* exhibits a wide geographic range extending into the northeastern United States.[33] Its ability to transmit DENV and CHIKV coupled with its aggressive biting behavior, suggest a potential role in ZIKV transmission, a hypothesis supported by the identification of ZIKV-positive *Ae. albopictus* pools during the 2007 Gabon outbreak.[4]

Previous studies of the vector competence of *Ae. albopictus* for ZIKV provided disparate results, with some finding populations of this species to be relatively poor vectors,[34] whereas others reported high susceptibility and transmission potential.[35,36] However, all of these previous studies used artificial bloodmeals, known to be less infectious than viremic animals for several arboviruses including ZIKV.[37] To better evaluate the potential role of *Ae. albopictus* in ongoing and future ZIKV outbreaks, we investigated its susceptibility to infection, dissemination, and potential for transmission following exposure to five strains of ZIKV. Because previous vector competency analyses indicate that mosquitoes from different geographic locations can differ in their susceptibility to arboviruses,[34] we used populations from ZIKV-epidemic locations, including two high-risk locations in the Americas, based on histories of DENV circulation[38]: the Rio Grande Valley, TX; Houston, TX; and Salvador, Brazil.

MATERIALS AND METHODS

Cells. Vero cells were purchased from American Type Tissue Culture Collection (Bethesda, MD) and maintained in Dulbecco's modification of Eagle's Medium (DMEM) (Invitrogen, Carlsbad, San Diego) supplemented with 5% fetal bovine serum (FBS) (Atlanta Biologicals, Norwalk, GA) and Penicillin/Streptomycin (P/S; 100 units/mL and 100 µg/mL, respectively) (Invitrogen) in a water-jacketed incubator at 37°C with 5% CO_2.

Viruses. The following ZIKV strains were used: FSS 130125 (GenBank accession no. KU955593), a human isolate from Cambodia (2010); DakAR 41525 (KU955591), an *Ae. africanus* isolate from Senegal (1984); MEX 1-7 (KX247632), isolated from an *Ae. aegypti* pool from Chiapas State, Mexico (2015); PRVABC 59 (KX377337), a human isolate derived from Puerto Rico (2015); and PB 81 (KU365780), an isolate derived from human blood from Brazil (2015). All viruses were acquired from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) at the University of Texas Medical Branch (UTMB) and passage histories are listed in Table 1. Stocks were titered by focus-forming assay as described below and frozen at −80°C in 30% FBS prior to mouse infections.

Mosquitoes. Adult female *Ae. albopictus* from the Rio Grande Valley (F5), Houston (F2), and Salvador, Brazil (F3), were housed in a 27 ± 1°C incubator (a typical mean temperature in tropical climates) with 80 ± 10% relative humidity, fed 10% sucrose ad libitum, and maintained at 16:8 light:dark photoperiod. Female mosquitoes in all experiments were fed 5 days posteclosion. Twenty-four hours prior to bloodmeals, sucrose was replaced with water, which was withdrawn 6 hours before feeding.

Murine infections. Four-week-old interferon type I receptor-knockout (A129) mice were infected intraperitoneally with 1×10^5 focus-forming units (FFUs) of each ZIKV strain, diluted in sterile phosphate buffered saline. This model generates predictable viremia at 1, 2, and 3 days postinfection to produce varied oral doses for mosquitoes.[39] One animal per day was randomly selected and anesthetized with 100 mg/kg of ketamine and placed on the screened lid of 0.5-L cardboard cartons containing sucrose-starved *Ae. albopictus*. Mosquitoes were allowed to feed for 30 minutes, then cold-anesthetized and fully engorged mosquitoes were incubated as described below. Following blood feeding, mice were killed and blood was collected for viremia assays as described below. All animal procedures and manipulations were approved by the UTMB Institutional Animal Care and Use Committee (IACUC).

Titrations. Mouse sera and positive saliva samples underwent 10-fold serial dilution in DMEM with 2% FBS and 1% P/S on 96 well plates; 100 µL of each dilution were then transferred to Vero cell monolayers on 24 well plates. After 1 hour at 37°C wells were overlaid with 0.8% methylcellulose in DMEM. Following 3 days incubation at 37°C, the overlay was removed and monolayers were rinsed twice with sterile Dulbecco's phosphate buffered saline

TABLE 1
Zika virus strain Information

Strain	Genbank accession	Origin	Location isolated	Year isolated	Passage history*
DakAR 41525	KU955591	*Aedes africanus*	Senegal	1984	AP61, C6/36 (2), Vero (3)
FSS 130125	KU955593	Human	Cambodia	2010	Vero, C6/36 (2), Vero (3)
MEX 1-7	KX247632	*Aedes aegypti*	Chiapas State, Mexico	2015	Vero (4), C6/36, Vero (3)
PB 81	KU365780	Human	Paraiba, Brazil	2015	Vero, C6/36, Vero
PRVABC 59	KX377337	Human	Puerto Rico	2015	Vero (5)

* Cells lines include AP61: *Aedes pseudoscutellaris* larvae; C6/36: *Aedes albopictus* larvae; Vero: *Chlorocebus aethiops* kidney epithelium.

(DPBS), and fixed for 1 hour at room temperature in ice-cold methanol:acetone (1:1). Detection of virus was conducted via focus-forming assay, as detailed below.

Mosquito infection, dissemination, and transmission potential. On days 3, 7, and 14 of incubation, 9–15 mosquitoes per group were cold-anesthetized, and legs removed and placed into microfuge tubes containing a steel ball bearing and 500 µL of DMEM, supplemented with 2% FBS, 1% P/S, and 2.5 µg/mL amphotericin B (Gibco, Waltham, MA). Mosquitoes were then restrained on a glass slide with mineral oil and their proboscis inserted for 30 minutes of salivation into a sterile 10 µL micropipette tip containing 8 µL of FBS, after which the expectorated saliva/FBS was added to 100 µL of DMEM supplemented with 1% P/S and 2.5 µg/mL amphotericin B. Mosquito bodies and legs were then triturated for 5 minutes at 26 Hz in a TissueLyser II (Qiagen, Venlo, the Netherlands). Following homogenization, samples were clarified by centrifugation at 200 × g for 5 minutes. Infections and detections were performed via the focus-forming assay described below.

Focus-forming assay. Focus-forming assays were performed as previously described,[40] with modifications. Briefly, viremic mouse sera or clarified mosquito samples were inoculated onto Vero cell monolayers on 24 or 96 well plates, respectively. After incubation for 3 days, media was removed and wells were washed. Plates were fixed using ice-cold methanol:acetone (1:1). Following complete air drying, plates were washed with PBS and then blocked with 3% FBS in PBS, followed by an overnight incubation with mouse hyperimmune serum against-ZIKV strain MR-766 (1:2,000 in blocking solution) (WRCEVA, UTMB). Plates were then washed with PBS followed by incubation with a goat anti-mouse secondary antibody conjugated to horseradish peroxidase (KPL, Gaithersburg, MD) diluted at 1:2,000 in blocking solution. Plates were washed with PBS, after which an aminoethylcarbazole solution (Enzo Diagnostics, Farmingdale, NY) prepared according to manufacturer's protocol, was added and plates were incubated in the dark. Development was halted by washing in tap water and plates were allowed to air dry at room temperature before scoring. For samples in 96 well plates, detection of any intracytoplasmic staining by light microscopy was defined as positive. For titrations of positive saliva samples and mouse serum on 24 well plates foci were counted either by eye or by light microscopy.

50% oral infectious dose calculation. The oral dose of a given ZIKV strain required to infect, disseminate, and transmit in a given sample of mosquitoes was calculated where possible, utilizing the method of Reed and Muench.[41]

Statistical analysis. The impact of mosquito strain, virus strain, and virus titer in the mouse and day of extrinsic incubation on infection in the mosquito body (a dichotomous yes/no variable) was analyzed using nominal logistic regression. Next, the impact of the factors listed earlier on dissemination into the legs from those mosquitoes with infected bodies, as well as secretion of virus into the saliva from those mosquitoes with a disseminated infection, was analyzed using nominal logistic regression. Saliva samples found positive in the 96 well format were titrated on 24 well plates and subjected to focus forming assay as described earlier. For samples that were positive in the initial screen but negative in the titration due to a titer below the limit of detection (10 FFU), a titer of 9 FFU was assigned to ensure conservative comparisons. Resultant titers were log-transformed, but since this failed to result in normal distribution titers were analyzed via a Wilcoxon test.

RESULTS

Comparison of ZIKV strains. *Rio Grande Ae. albopictus.* With respect to our analyses, it should be noted that ideally, the virus strains tested in all three populations of mosquitoes would be matched, so as to facilitate easier comparisons. However, on availability, we replaced DakAR 41525 and FSS13025 with modern epidemic strains PRVABC 59 and PB 81 due to their increased public health relevance. Therefore, because virus and mosquito strains were not completely blocked, we could not compare all virus strains among all mosquito strains. Thus we first compared the impact of virus strain, viremia titer in the mouse, and day of extrinsic incubation, as well as interactions among all three factors, on infection of Rio Grande mosquitoes, which fed on MEX 1-7-, FSS 133205-, and DakAR 41525-infected mice. Substantially higher viremia titers were generated in mice infected by the DakAR 41525 strain compared with the other two (Figure 1). We found no significant interactions among the three factors, and a significant effect only of virus strain (df = 2, χ^2 = 7.07, P = 0.029), with DakAR 41525 and MEX 1-7 infecting a significantly higher proportion of mosquitoes than FSS 13025. This effect was driven primarily by the greater infectivity of DakAR 41525 and MEX 1-7 at the lower viremia titers. We then compared the effect of the three factors on ZIKV dissemination in infected mosquitoes. This analysis revealed a significant interaction between viral strain and days of extrinsic incubation (df = 2, χ^2 = 10.5, P = 0.005), but no significant interactions among the remaining factors. We therefore conducted a simple effects test of the impact of viral strain and viremia titer for each day of extrinsic incubation (3, 7, 14) individually. There were very few disseminated infections on day 3, precluding comparison, but on days 7 and 14 there were significant effects of both virus strain and viremia titer ($P \leq 0.001$ for all comparisons). Virus dissemination increased with increasing viremia titer, and at a comparable viremia titer, the DakAR 41525 strain of ZIKV (1.6×10^6 FFU/mL) disseminated more efficiently than the FSS 13025 (3.5×10^6 FFU/mL) or MEX 1-7 (1.0×10^6 FFU/mL) strains. Finally, we compared the efficiency of dissemination by different ZIKV strains following different viremia titers and days of extrinsic incubation. This analysis detected a significant interaction between viremia titer and days of extrinsic incubation (df = 2, χ^2 = 12.8, P = 0.002). Thus we conducted a simple effects test of the impact of virus strain and titer at days 7 and 14 of extrinsic incubation (no dissemination was detected at day 3 of extrinsic incubation). This analysis detected no significant differences at day 7 but a significant interaction of virus titer and strain on day 14, with virus secretion into the saliva increasing with viremia titer, and with the DakAR 41525 strain of ZIKV generating a higher percentage of infectious saliva than the other two strains. Overall, these results indicated that the DakAR 41525 disseminated more efficiently and was shed into the saliva more frequently in Rio Grande Valley *Ae. albopictus* compared with the MEX 1-7 and FSS 13025 ZIKV strains.
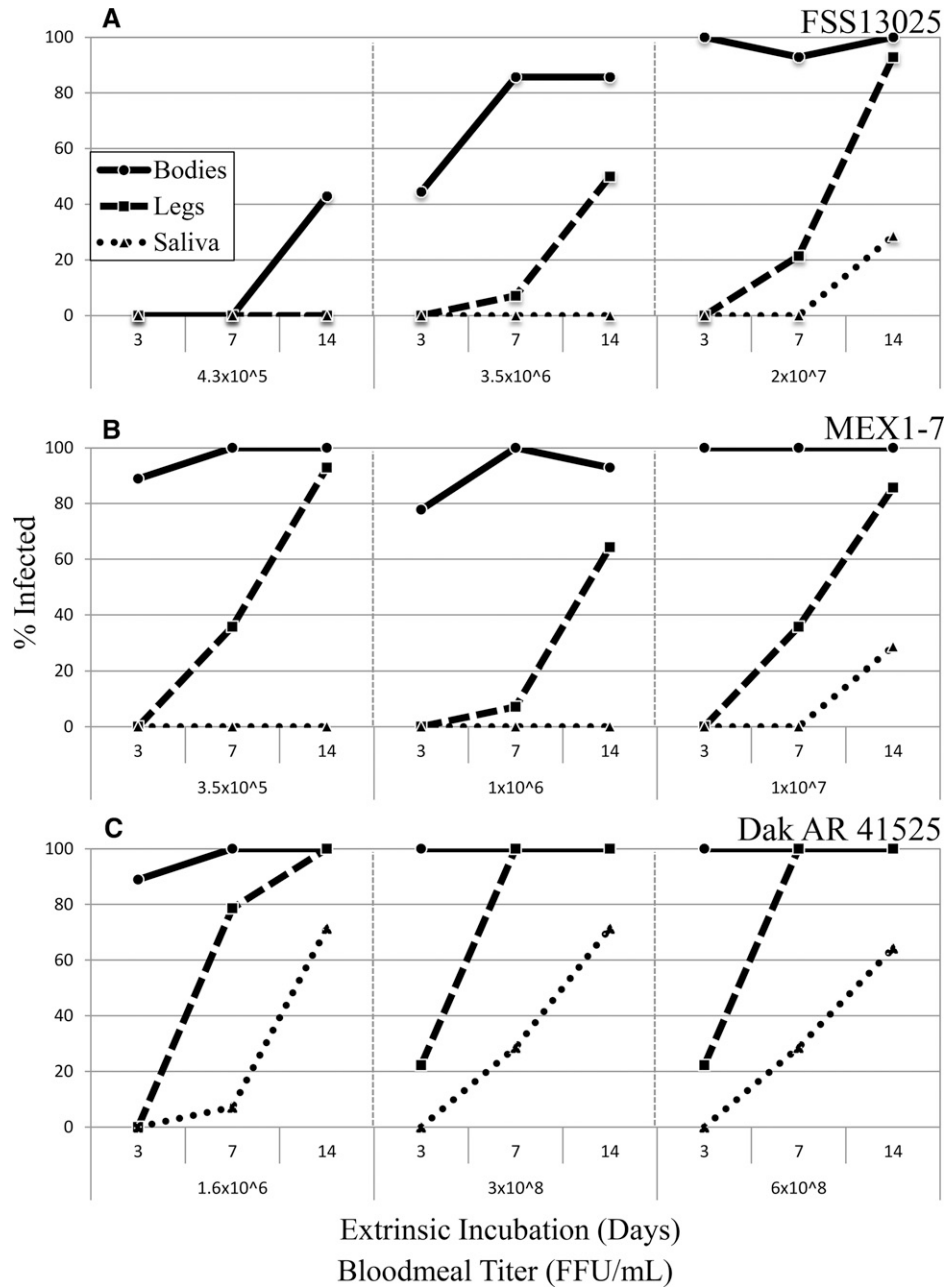
FIGURE 1.   Infection, dissemination, and potential transmission of three Zika virus strains by *Aedes albopictus* from the Rio Grande Valley, TX, following bloodmeal from viremic A129 mice infected with (**A**) FSS 13025 (Cambodia, 2010), (**B**) MEX 1-7 (Mexico, 2015) or (**C**) DakAR 41525 (Senegal 41525) and assays on day 3 (N = nine mosquitoes per virus), day 7 (N = 14), and day 14 (N = 14).

Houston Ae. albopictus. A similar analysis to the one described earlier detected no significant interactions or main effects of virus strain, virus titer, or days of extrinsic incubation on infection of Houston mosquitos exposed to PB 81, MEX 1-7, and PRVABC 59 ZIKV strains (Figure 2). This analysis detected no significant interactions or main effects of virus strain, virus titer, or days of extrinsic incubation on infection. Analysis of dissemination from infected bodies revealed a three-way interaction among the independent variables. A simple effects test of virus strain at viremia titer = 7.0 ± 0.2 log$_{10}$ FFU/mL and day 14 of extrinsic incubation showed no differences among the three ZIKV strains. There were no interactions among the three factors on secretion of virus into saliva and only virus strain influenced this outcome, with PRVABC 59 ZIKV shed into saliva more efficiently than the other two strains (df = 2, χ^2 = 17.2, P = 0.0002), which failed to produce detectable virus in saliva. Overall, ZIKV strain PRVABC 59 was more efficiently shed into the saliva of Houston *Ae. albopictus* compared with PB 81 and MEX 1-7 (Figure 2).

Salvador Ae. albopictus. Only days of extrinsic incubation significantly influenced infection or dissemination of PB 81 and MEX 1-7 in Salvador mosquitoes (P < 0.003 for both comparisons). Neither virus strain was detected in mosquito saliva (Figure 3).

Comparison of mosquito strains. *MEX 1-7 ZIKV.* Using the same data set that we used to compare virus strains, we next compared the effect of mosquito strain, virus titer,
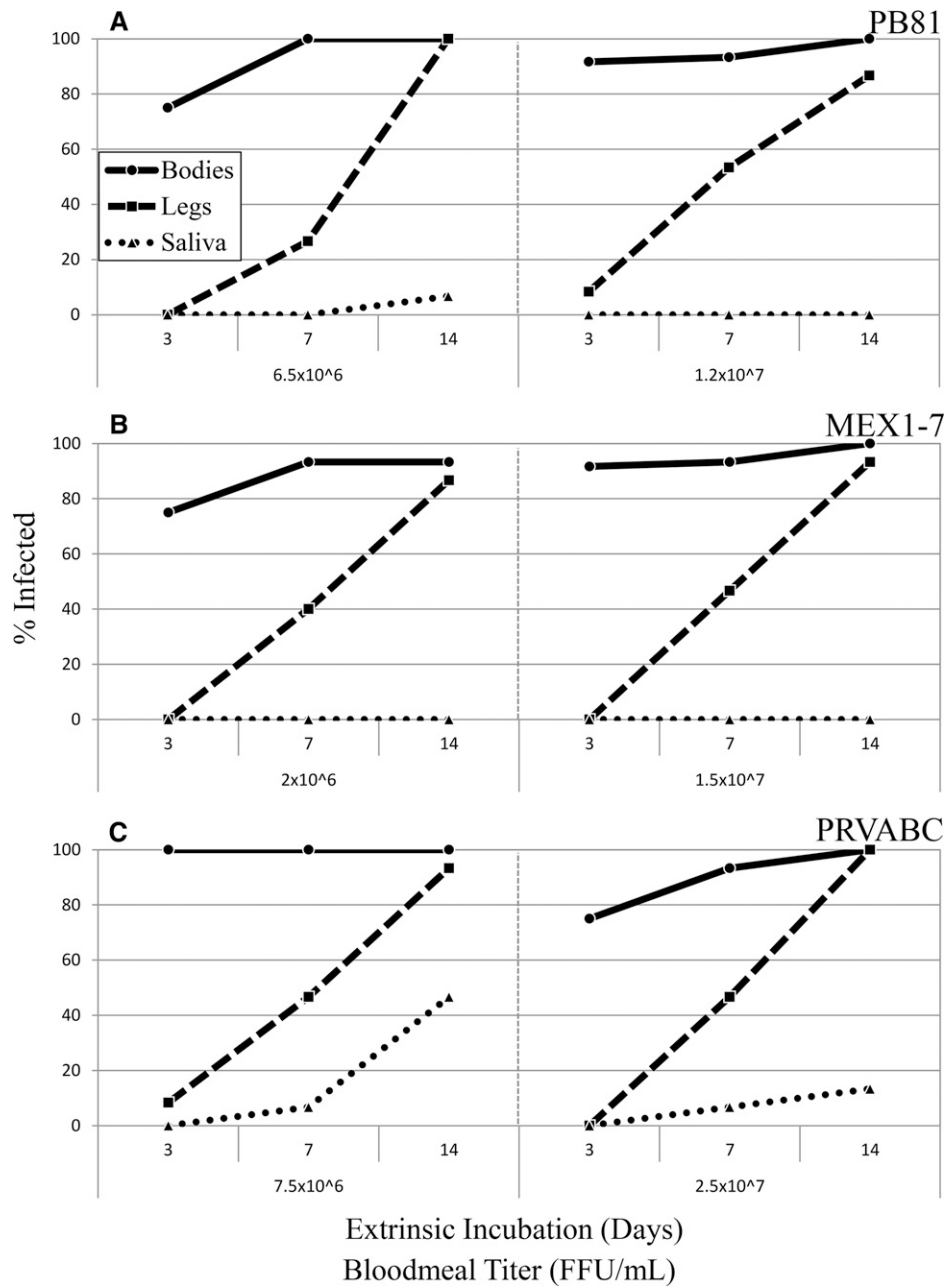
FIGURE 2. Infection, dissemination, and potential transmission of three Zika virus strains by *Aedes albopictus* from Houston, TX, following bloodmeal from viremic A129 mice infected with (**A**) PB 81 (Brazil, 2015), (**B**) MEX 1-7 (Mexico, 2015) or (**C**) PRVABC 59 (Puerto Rico 2015) and assays on day 3 (*N* = 12 mosquitoes per virus), day 7 (*N* = 15), and day 14 (*N* = 15).

and days of extrinsic incubation on infection by MEX 1-7, the only ZIKV strain fed to all three *Ae. albopictus* populations. We detected a significant interaction between virus titer and mosquito strain (df = 2, χ^2 = 6.48, P = 0.039). We therefore proceeded to conduct a simple effects test of the impact of mosquito strain and days of extrinsic incubation with a viremia titer of 7.0 ± 0.2 $\log_{10}$ FFU/mL. This comparison revealed no significant interaction between the two factors and a significant effect only of mosquito strain (df = 2, χ^2 = 43.7, P < 0.0001), with Houston and Rio Grande mosquitoes showing significantly higher susceptibility than Salvador mosquitoes. For dissemination from infected mosquitoes, we detected no significant interactions among

factors, with a significant impact of both mosquito strain (df = 2, χ^2 = 16.7, P < 0.0002) and days of extrinsic incubation (df = 1, χ^2 = 59.6, P < 0.0001). As expected, dissemination increased as extrinsic incubation increased. Additionally, dissemination was significantly lower in the Salvador strain of *Ae. albopictus* than in the two U.S. strains. MEX 1-7 produced detectable virus in the saliva of only the Rio Grande mosquitoes. Overall, only *Ae. albopictus* from the Rio Grande Valley proved competent for transmission potential of MEX 1-7. However, *Ae. albopictus* from Houston proved more susceptible to disseminated infections of ZIKV strain MEX 1-7 when compared with mosquitoes from Salvador, Brazil.
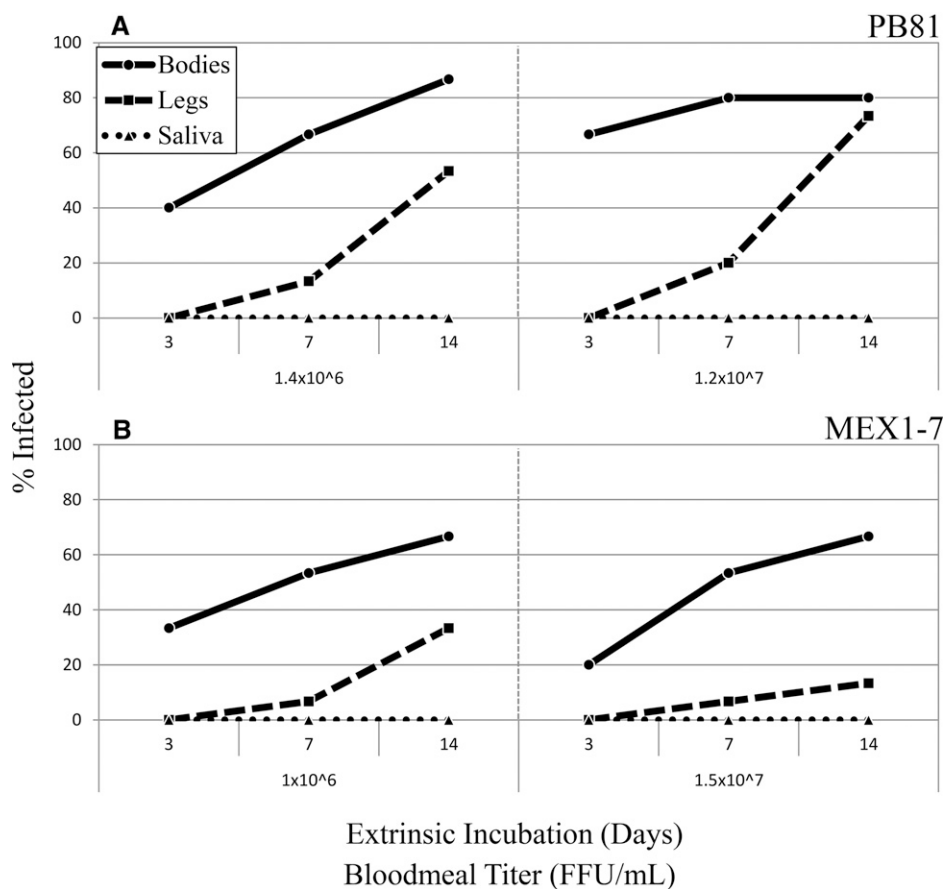
FIGURE 3. Infection, dissemination, and transmission of two Zika virus strains by *Aedes albopictus* from Salvador, Brazil, following blood-meal from viremic A129 mice infected with (**A**) PB 81 (Brazil, 2015) or (**B**) MEX 1-7 (Mexico, 2015) and assays on day 3 (N = 15 mosquitoes per virus), day 7 (N = 15), and day 14(N = 15).

PB 81 ZIKV. We then compared the infectivity of ZIKV strain PB 81 in the two mosquito strains tested: Houston and Salvador. We detected a significant interaction between mosquito strain and days of extrinsic incubation (df = 2, χ^2 = 4.8, P = 0.03), so we conducted a simple effects test of the impact of mosquito strain and virus titer for each day of extrinsic incubation, individually. This analysis revealed a significant effect of mosquito strain on days 7 and 14 ($P < 0.03$ for both comparisons), with Houston mosquitoes showing significantly greater susceptibility than Salvador mosquitoes. Dissemination among infected mosquitoes was shaped by a significant three-way interaction among mosquito strain, virus titer, and days of extrinsic incubation, with the general pattern that dissemination increased with virus titer and days of extrinsic incubation, but dissemination was generally higher in Houston mosquitoes. Virus was detected in the saliva of only the Houston mosquitoes. Overall, *Ae. albopictus* from Houston were more competent for transmission of ZIKV strain PB 81 compared with the Salvador, Brazil, population.

Saliva titers. Because *Ae. albopictus* from the Rio Grande Valley and Houston were fed on different sets of ZIKV strains, it was not possible to compare the two mosquito populations. Instead, we compared the saliva titers of three viruses, DakAR 41525, FSS 13025, and MEX 1-7, in Rio Grande mosquitoes that were fed on viremia titers of 8.5, 7.3, and 7.0 $\log_{10}$ FFU/mL, respectively, and sampled on day 14 of extrinsic incubation. All three viruses reached median saliva titers of 1.0 $\log_{10}$ FFU per collection sample and mean titers of 2.1, 1.5, and 1.6 $\log_{10}$ FFU per collection sample in saliva, respectively, which did not differ significantly (Wilcoxon, df = 2, N = 18, P = 0.78). We then compared the saliva titers in Rio Grande mosquitoes fed on the DakAR 41525 strain of ZIKV at three different viremia titers (6.2, 8.5, 8.8 $\log_{10}$ FFU/mL, respectively) and sampled at day 14 of extrinsic incubation; these titers (3.3, 3.7, and 3.6 $\log_{10}$ FFU per collection sample) also did not differ significantly (Wilcoxon test, df = 2, N = 29, P = 0.74). Titers generally increased between days 7 and 14 of extrinsic incubation, but the large number of negative day 7 samples precluded analysis of titer as a continuous variable.

50% oral infectious doses. The 50% oral infectious dose (OID_{50}) values were interpolated utilizing the method of Reed and Muench when infection encompassed 50% at the doses used in the study. For a majority of *Ae. albopictus* populations fed on ZIKV strains the OID_{50} could not be calculated due to infection rates exceeding 50%, even at the lowest tested viremia titers (e.g., DakAR 41525). The FSS 13025 strain demonstrated an OID_{50} of 6.7 $\log_{10}$ FFU/mL for assay on day 7 of extrinsic incubation which decreased to 5.9 $\log_{10}$ FFU/mL by day 14 of extrinsic incubation in Rio Grande population of *Ae. albopictus*. PB 81 exhibited an OID_{50} of 6.8 $\log_{10}$ FFU/mL for assay on day 3 of extrinsic incubation in Salvador *Ae. albopictus* (Supplemental Table 1).

DISCUSSION

These results demonstrate the vector competence of *Ae. albopictus* from various geographic locations in the Americas for multiple strains of ZIKV, an important component of establishing risk and designing control strategies. To determine the role that *Ae. albopictus* may play in outbreak settings (vectorial capacity), additional factors such as range, longevity and feeding behaviors, especially as compared with the domestic and highly anthropophilic *Ae. aegypti* must be considered. *Aedes albopictus* is widespread throughout many temperate regions of the United Ststes where *Ae. aegypti* is not typically found, such as the upper Midwest and the northeast.[11,29,31] Anthropophilic and endophilic feeding behaviors of *Ae. aegypti*, however, make the species more apt at transmitting human arboviruses when compared with the more opportunistic and exophilic bloodfeeding behavior of *Ae. albopictus*.[29,42] Additionally, the tendency of *Ae. aegypti* to take multiple bloodmeals per gonotrophic cycle means these mosquitoes are more likely to become infected and more likely to transmit to multiple people once infected compared with *Ae. albopictus* mosquitoes.[42] ZIKV has been detected in both species during outbreaks, *Ae. albopictus* in Gabon in 2007[4] and *Ae. aegypti* in Mexico in 2015[27] and Brazil in 2016,[28] indicating that each species can play a role in ZIKV epidemics.

As demonstrated in previous studies with both *Ae. aegypti* and *Ae. albopictus*, we found that vector competence of *Ae. albopictus* varies with the geographic origin of both vector and virus strain. To more accurately reflect natural infection, we used viremic A129 mice, previously shown to be more infectious for mosquitoes than artificial bloodmeals. For example, when *Ae. aegypti* from Salvador, Brazil, were orally infected with 6 $\log_{10}$ FFU/mL of ZIKV (FSS 13025) by artificial bloodmeal, by day 14 of extrinsic incubation, 75% of mosquitoes were infected, with 67% of those infections disseminating, but never with virus detected in the saliva. When the same population of mosquitoes was infected via a viremic A129 mouse circulating 6 $\log_{10}$ FFU/mL, on day 14 of extrinsic incubation all tested mosquitoes were infected, with 92% of infections disseminating and 61% of the disseminated infections reaching the saliva.[37] This pronounced difference has been observed with other arboviruses such as western equine encephalitis virus, and is at least partly explained by clotting of blood ingested from an animal resulting in greater viral concentration directly adjacent to the mosquito midgut epithelium.[43] The flaviviruses, St. Louis encephalitis virus and DENV also exhibit reduced infectivity for *Culex quinquefasciatus* Say and *Ae. aegypti*, respectively, when freeze-thawed virus is compared with virus freshly harvested from cell cultures, even when matched for final infectious titer.[44] Recently, the reduction of infectivity as a result of freeze-thaws was also demonstrated for ZIKV infection of *Ae. aegypti*.[45] All of these studies support the use of viremic animals for accurately assessing vector competence.

Strikingly, our findings with *Ae. albopictus* from the Rio Grande Valley of Texas corroborate field findings in Gabon during the 2007 outbreak.[4] When Rio Grande Valley mosquitoes were exposed to the African lineage ZIKV strain DakAR 41525 at 6 and 8 $\log_{10}$ FFU/mL, they were uniformly infected and developed disseminated infections, such that by day 14 of extrinsic incubation, 60% of mosquitoes had shed virus into their saliva (Figure 1C). Phylogenetically, the Gabon 2007 strain clusters closely with the 1984 Senegal ZIKV strain (DakAR 41525) based on envelope and NS3 genes.[4] Additionally, recent studies[45] as well as our own data[37] have also demonstrated efficient transmission of African lineage ZIKV strains by American *Ae. aegypti*. In sum, these data suggest that the African lineage of ZIKV is well adapted for urban transmission by both *Ae. aegypti* and *Ae. albopictus*.

A limitation of our understanding of vector competence for ZIKV is the dearth of data for infectious human viremia profiles, with titers ranging from 0.49 to 3.39 $\log_{10}$ infectious particles/mL or 900 to 729, 000 RNA copies/mL,[24,46,47] orders of magnitude below the viremia titers to which mosquitoes in our analyses were exposed, and well below our estimated OID$_{50}$ of both FSS 13025 and PB 81 in *Ae. albopictus* from the Rio Grande and Salvador, Brazil, respectively. Another limitation is that previous studies found that colonization of *Ae. aegypti* and *Ae. albopictus* alters their competence for DENV.[48,49] However, our use of low-generation colonies should have minimized these potential artifacts. Also, ideally our mosquito and virus strains would have been matched, and all possible combinations would have been tested. This was not possible because of logistical constraints and the acquisition of virus and mosquito strains after the study was initiated. For example, on availability, we replaced DakAR 41525 and FSS13025 with recent epidemic strains PRVABC 59 and PB 81 due to their increased public health relevance.

Our data strongly demonstrated that, while all tested populations of *Ae. albopictus* proved susceptible to midgut and disseminated infections at varying efficiencies, Houston and Salvador mosquitoes were relatively incapable of transmitting ZIKV. In order for an arbovirus to be transmitted by a competent vector, it must 1) be ingested via a bloodmeal from an infected host; 2) infect epithelial cells of the mosquito midgut; 3) disseminate from the midgut into the hemocoel and infect further tissues; 4) infect the salivary glands; and 5) be shed into acinar cavities for innoculation into a new host upon subsequent feedings.[50,51] The relative inability of Houston and Salvador *Ae. albopictus* to transmit ZIKV strains, despite the presence of disseminated virus in hemocoel, suggests the possibility of a salivary gland infection or salivary egress barrier.[52] In the case of the former, ZIKV may be failing to infect secondary amplification tissues such as the fat bodies, hemocytes, nerve, or muscle tissues following midgut escape, preventing sufficient replication to efficiently infect the salivary glands. Anatomic analyses utilizing ZIKV reporter systems[53] in transmission-competent versus transmission-incompetent *Ae. albopictus* populations are needed to address these hypotheses.

The explosive spread of ZIKV throughout tropical and subtropical regions of the Americas has raised concerns that mosquitoes other than *Ae. aegypti* may be transmitting ZIKV.[54] In our study, *Ae. albopictus* from Salvador, Brazil, orally exposed to two American strains of ZIKV (MEX 1-7 and PB 81) at high titers (6 or 7 $\log_{10}$ FFU/mL) shed no virus into saliva, even by day 14 of extrinsic incubation. Although we tested only one Brazilian mosquito population, the lack of transmission competence in the population tested with high viremia titers, coupled with a lack of field data from Mexico and Brazil reporting ZIKV positive *Ae. albopictus* pools, calls a significant role for this species into question, especially when taken together with previous

reports on the competency of *Ae. albopictus* from Jurjuba, Rio de Janeiro. Of the seven populations of mosquitoes (all of which were populations from the Americas, five *Ae. aegypti* populations and two *Ae. albopictus* populations) tested in that study, the Jurjuba *Ae. albopictus* proved to be the least susceptible, although the transmission potential of this population was not tested.[45]

A critical component of viral pathogenesis is the infectious dose, or the saliva titer for arboviruses.[55] We found a range of saliva titers, with a maximum titer of 3.72 $\log_{10}$ FFU/collection of DakAR 41525 in a Rio Grande Valley mosquito sample. Analyses in other arboviruses have reported a large variation in saliva titers dependent on the virus and vector in question. For example, when the *alphavirus* Venezuelan equine encephalitis virus (VEEV) was used to infect *Ae. albopictus* and *Aedes taeniorhynchus* (Wiedemann) and saliva was collected in vitro, the resultant titers ranged from 0.2 to 1.1 $\log_{10}$ and 0.2 to 3.2 $\log_{10}$ plaque forming units (PFU) per collection, respectively.[56] Further analyses comparing in vitro saliva collection of VEEV to in vivo inoculation revealed that artificial salivation (mean 74 PFU) overestimates the in vivo inoculum by nearly 10-fold (mean 11 PFU).[57] Similar analyses of in vivo saliva deposition of WNV from four different species of mosquitoes (*Culex tarsalis* Coquillet, *Culex pipiens* Linnaeus, *Aedes japonicus* [Theobald], and *Aedes triseriatus* [Say]) into a murine host yielded a range of saliva titers (3.4–6.1 $\log_{10}$ PFU),[58] whereas a separate analysis of WNV utilizing saliva collected from *Cx. tarsalis in vitro* yielded a titer of 1.41 $\log_{10}$ PFU.[59] Further complicating in vitro collection of mosquito saliva is the finding that mosquitoes demonstrate host-seeking behavior until imbibing 2.5–3.5 μL of blood.[60] In vitro saliva collections (our own as well as previous reports[37,57]) comprise minimal volumes (≤ potential for mosquitoes to imbibe a significant percentage of expectorated saliva). To account for potential volume loss, we report our results as FFU per collection, as opposed to FFU/mL. In summary, disparate methodologies of saliva collection from different mosquito populations infected with different viruses limit what conclusions can be drawn from salivary viral titers and underscore the necessity for standardization of methodologies.

Like other vector competence studies,[34] our data suggest significant variation as a function of mosquito origin and viral strain. Laboratory competency studies have produced disparate findings, with *Ae. albopictus* populations being shown to be both poor[33] and relatively competent vectors.[35,36] These disparities underscore the need for further studies in both the laboratory and the field to determine the potential role that *Ae. albopictus* may play in future ZIKV outbreaks, especially in temperate climates where *Ae. aegypti* cannot survive cold winters. Variables among this and other vector competence studies reported in the literature, such as colonization, mosquito microbiome composition, and genetics differences, should be further explored to determine their impact on ZIKV transmission.

Authors' addresses: Sasha R. Azar, Christopher M. Roundy, and Scott C. Weaver, Department of Pathology, Institute for Human Infections and Immunity, University of Texas Medical Branch, Galveston, TX, Center for Tropical Diseases, University of Texas Medical Branch, Galveston, TX, and Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX, E-mails: srazar@utmb.edu, cmroundy@utmb.edu, and sweaver@utmb.edu. Shannan L. Rossi, Jing H. Huang, Grace Leal, Ruimei Yun, and Scott C. Weaver, Department of Pathology, Institute for Human Infections and Immunity, University of Texas Medical Branch, Galveston, TX, and Center for Tropical Diseases, University of Texas Medical Branch, Galveston, TX, E-mails: slrossi@utmb.edu, jhhuang@utmb.edu, grleal@utmb.edu, ruyun@utmb.edu, and nivasila@utmb.edu. Ildefonso Fernandez-Salas, Instituto Nacional de Salud Pública, Centro Regional de Salud Pública, Tapachula, Chiapas, México, E-mail: ildefonso.fernandez@insp.mxhiapas. Christopher J. Vitek, University of Texas Rio Grande Valley, Edinburg, TX, E-mail: christopher.vitek@utrgv.edu. Igor A. D. Paploski and Guilherme S. Ribeiro, Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Ministério da Saúde, Candeal, Salvador, Brazil, and Instituto de Saúde Coletiva, Universidade Federal da Bahia, Salvador, Brazil, E-mails: igorufprmv@gmail.com and gsribeiro@gmail.com. Pamela M. Stark, Jeremy Vela, Mustapha Debboun, and Martin Reyna, Harris County Public Health Mosquito and Vector Control Division, Houston, TX, E-mails: pstark@hcphes.org, jvela@hcphes.org, mdebboun@hcphes.org, and mreyna@hcphes.org. Uriel Kitron, Population Biology, Ecology, and Evolution Graduate Program, Graduate Division of Biological and Biomedical Sciences, Department of Environmental Studies, Emory University, Atlanta, GA, E-mail: ukitron@emory.edu. Kathryn A. Hanley, Department of Biology, New Mexico State University, Las Cruces, New Mexico, E-mail: khanley@nmsu.edu.

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