

## *Anopheles albimanus* is a Potential Alphavirus Vector in the Americas

Gerard Terradas,<sup>1,2,3</sup> Mario Novelo,<sup>1,2,3</sup> Hillery Metz,<sup>1,2,3</sup> Marco Brustolin,<sup>1,2,3</sup> and Jason L. Rasgon<sup>1,2,3\*</sup>

<sup>1</sup>Department of Entomology, The Pennsylvania State University, University Park, Pennsylvania; <sup>2</sup>Center for Infectious Disease Dynamics, The Pennsylvania State University, University Park, Pennsylvania; <sup>3</sup>Huck Institutes of the Life Sciences, The Pennsylvania State University, University Park, Pennsylvania

**Abstract.** Despite its ecological flexibility and geographical co-occurrence with human pathogens, little is known about the ability of *Anopheles albimanus* to transmit arboviruses. To address this gap, we challenged *An. albimanus* females with four alphaviruses and one flavivirus and monitored the progression of infections. We found this species is an efficient vector of the alphaviruses Mayaro virus, O'nyong-nyong virus, and Sindbis virus, although the latter two do not currently exist in its habitat range. *An. albimanus* was able to become infected with Chikungunya virus, but virus dissemination was rare (indicating the presence of a midgut escape barrier), and no mosquito transmitted. Mayaro virus rapidly established disseminated infections in *An. albimanus* females and was detected in the saliva of a substantial proportion of infected mosquitoes. Consistent with previous work in other anophelines, we find that *An. albimanus* is refractory to infection with flaviviruses, a phenotype that did not depend on midgut-specific barriers. Our work demonstrates that *An. albimanus* may be a vector of neglected emerging human pathogens and adds to recent evidence that anophelines are competent vectors for diverse arboviruses.

### INTRODUCTION

More than 400 species have been described for the genus *Anopheles*, with approximately 40 regarded as vectors of interest,<sup>1</sup> mostly of *Plasmodium* parasites. Anopheline mosquitoes are primarily known for transmitting malaria,<sup>2</sup> but they also have the potential to transmit viruses.<sup>3,4</sup> In general, they are highly mobile and thrive by using human activities and movement to disperse around the globe.<sup>5,6</sup> Although the most known and well-studied species are the African *Anopheles gambiae* and the Indo-Iranian *Anopheles stephensi*, due to the malaria-associated socioeconomic and health burdens they cause in those regions,<sup>2</sup> less well studied anopheline species predominate in other areas of the planet with the potential to spread different pathogens.

*Anopheles albimanus* is the main anopheline inhabiting northern South America, Central America (reviewed in detail in reference 7), and the Caribbean islands.<sup>8</sup> Its broad geographical distribution may be aided by the species' ability to survive in both fresh<sup>9,10</sup> and brackish water.<sup>11,12</sup> Although it remains incompletely understood, *An. albimanus* has been described as a much more zoophilic, crepuscular, and exophagic mosquito with higher adaptive capabilities than other anopheline species,<sup>13–16</sup> all of which affect the species' success in transmitting *Plasmodium*. However, host availability and environmental conditions appear to influence its host choice<sup>17,18</sup> and resting behavior.<sup>19</sup> The flexible behavior of this species may be facilitating its spread into the southern United States, lower latitudes (more temperate areas) in South America, and urban and peri-urban settlements where it encounters human hosts. Its expansion may also be helped by climate change, which is broadening the species' geographical habitat range.<sup>8</sup> Despite the work that has been done to characterize its ecology and behavior, little is known about the capacity of the species to harbor and transmit classic and emerging tropical mosquito-borne viruses.

Many arboviruses produce similar disease symptoms in humans that include fever, headache, rash, diarrhea, and joint pain, which can last for months. Because treatment is not specific to the etiological agent and neither are many clinical surveillance and diagnostic tools, the prevalence of emerging viruses can be misdiagnosed and hence underestimated in areas with more common viral outbreaks such as Chikungunya or dengue viruses, as initially occurred with the Zika virus epidemic.<sup>20</sup> However, despite displaying similar clinical symptoms, viruses may differ in intrinsic replication rates or use different cellular receptors in the mosquito to achieve a successful human-to-mosquito-to-human viral transmission route. Arboviruses rely on the rapid infection of a mosquito after feeding on an infectious host, and must penetrate and overcome multiple tissue and immune barriers to propagate throughout the body and reach the salivary glands.<sup>21</sup> The virus must also replicate in the salivary glands efficiently to later infect a naive vertebrate host through salivation during a second bloodmeal. To date, only one arbovirus is known to be primarily transmitted through the bite of *Anopheles* mosquitoes in the field (O'nyong-nyong virus<sup>22</sup>; *Anopheles funestus* and *An. gambiae*), but other *Anopheles* species have been shown to be capable vectors of alphaviruses in the laboratory.<sup>23,24</sup>

Here, we ask whether *An. albimanus* is a competent vector of arboviruses. We orally challenged adult females with infectious bloodmeals containing one of the following togaviruses (genus *Alphavirus*): Mayaro (MAYV; -D and -L genotypes), Chikungunya (CHIKV; Asian lineage), O'nyong-nyong (ONNV), and Sindbis virus (SINV), as well as the flavivirus dengue virus serotype 2 (DENV-2; Asian lineage), due to its prevalence and health impact worldwide. Collectively, these togaviruses produce human disease that spans the planet (Figure 1A): MAYV in Central/South America, ONNV in Africa, CHIKV across the tropics, and SINV in colder climates. The tested viruses have differences in both virion structure ( $T = 3$  [flavivirus] versus  $T = 4$  [alphavirus] icosahedral geometry, lack of M structural protein in alphaviruses) and envelope proteins that create variation in their capacity for cellular entry and invasion, as well as differences in genomic structure (flaviviruses do not have a poly-A 3' end) (Figure 1B). Following viral challenge, we monitored mosquitoes for arbovirus

\*Address correspondence to Jason Rasgon, Department of Entomology, The Pennsylvania State University, Millennium Science Complex, Rm. W127, University Park, PA 16802. E-mail: jlr54@psu.edu

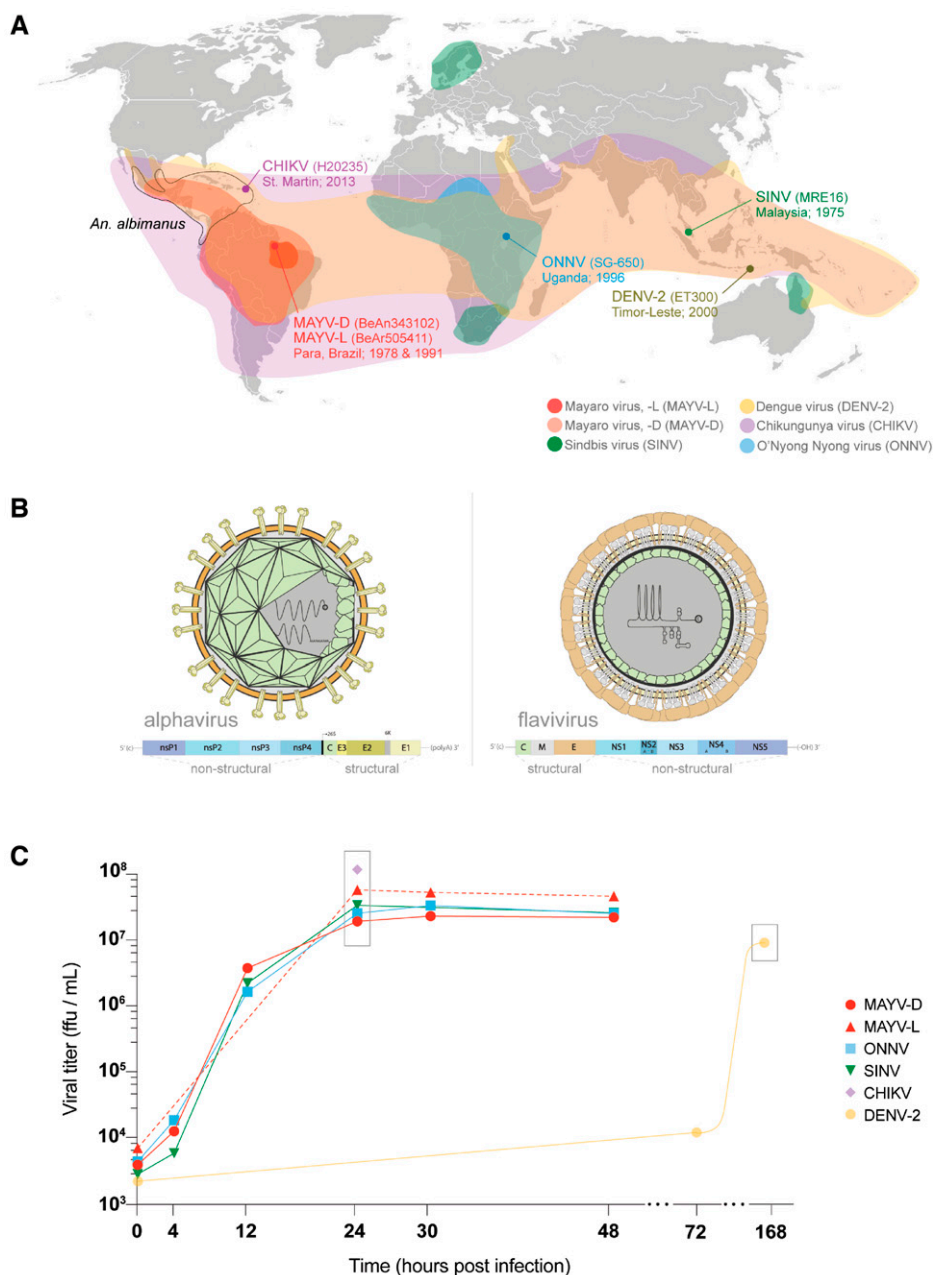


FIGURE 1. (A) Geographical distribution of the arboviruses assessed in this study mapped with the geographical range of the Caribbean mosquito vector *An. albimanus* (black). The place of precedence and time of collection are noted for each tested virus. (B) Virion and genomic structure of alphaviruses (MAYV, ONNV, CHIKV, SINV) and flaviviruses (DENV). (C) Alphavirus (up to 48 hours) and flavivirus (up to 7 days) stocks were grown using Vero or C6/36 cells, respectively. Growth curves depict increasing infectious viral loads over time, from inoculation to final collection. The titers used for mosquito challenge experiments are shown within grey boxes.

infection, dissemination of virus beyond the midgut and throughout the body, and secretion of virus in saliva. We report for the first time that *An. albimanus* can become infected with and transmit multiple alphaviruses—including MAYV, a human pathogen that is already spreading within this mosquito's geographic range in the Americas.

## RESULTS

**Growth kinetics of arboviruses in vitro.** Prior to in vivo infection experiments, we assessed the in vitro growth of each virus over time. Each virus was propagated in Vero

(alphaviruses) or C6/36 (flavivirus) cells. Alphaviruses typically replicate more quickly than flaviviruses, which can be seen in the rapid and severe cytopathic effects they produce in invaded cells of vertebrate origin.<sup>25</sup> To ensure virus for subsequent experiments was harvested during its replicative stage, we collected samples for alphaviruses across different time points up to 48 hours postinfection (hpi), when the presence of abundant cellular death and abnormal media pH were visibly evident. Infectious titers were assessed by plaque-forming assay (PFA; SINV) or focus-forming assay (FFA; the remaining viruses). All alphavirus titers peaked at 24 hpi with viral titers  $> 10^7$  focus-forming units (ffu) (or

plaque-forming units; pfu) per milliliter (Figure 1C), after which they declined only slightly until the final time point (48 hpi). Note that CHIKV's viral titer ( $3 \times 10^8$  ffu/mL) was assessed only at 24 hpi due to biosafety level 3 (BSL-3) laboratory constraints. Conversely, DENV-2 infections were sampled at days 0, 3, and 7, and we found viral titers in supernatant were highest at day 7 ( $1 \times 10^7$  ffu/mL).

**Both MAYV genotypes infect, replicate, and are transmitted efficiently by *An. albimanus*.** For MAYV mosquito challenges, we used two different strains of the virus (BeAn343102, genotype -D; BeAr505411, genotype -L). Results are reported as 1) infection rate (IR), the proportion of challenged mosquitoes with infected midguts, 2) dissemination rate (DR) and efficiency (DE), the proportion of infected (DR) or challenged (DE) mosquitoes with infected bodies, and 3) transmission rate (TR) and efficiency (TE), the proportion of infected (TR) or challenged (TE) mosquitoes with infected saliva. These ratios were calculated using the number of samples deemed infection positive by viral titers measured from the midgut, carcass (rest of the body), and saliva, respectively, and are reported with numerical subscripts to indicate sampling day. Viral titers are reported as means at individual time points (shown as  $M_x$ ,  $C_x$ , or  $S_{x[in]}$ , where "M" represents midgut, "C" represents carcass, "S" represents saliva samples, "x" denotes the day of collection (days post-infection; dpi), and "in" represents only infected subsets. Average titers across all time points are reported without a time point subscript.

For both MAYV genotypes, we found that *An. albimanus* was highly susceptible to infection, dissemination, and transmission. MAYV-D successfully established infections in the midgut and disseminated to the rest of the body in nearly all mosquitoes at all surveyed time points (Figure 2A, Table 1; IR: 97.6%; DR: 97.6%; DE: 95.3%). Although infection prevalence was consistent across time, disseminated viral titers rose until 10 dpi (Figure 2A;  $C_7$  versus  $C_{10}$ :  $U = 250$ ,  $P = 0.034$ ), when both infection ( $M_{10}$ :  $1.3 \times 10^9$  ffu/mL) and dissemination ( $C_{10}$ :  $3.5 \times 10^7$  ffu/mL) titers were highest. The infection and dissemination patterns of MAYV-L were similar to those of MAYV-D, with the virus infecting and disseminating through mosquitoes at high rates (Figure 2B, Table 1; IR: 94.3%; DR: 98.8%; DE: 93.2%). As with MAYV-D, MAYV-L titers also peaked at 10 dpi both in midguts ( $M_{10}$ :  $7.6 \times 10^6$  ffu/mL;  $M_7$  versus  $M_{10}$ :  $U = 125$ ,  $P < 0.0001$ ) and carcasses ( $C_{10}$ :  $2.5 \times 10^6$  ffu/mL;  $C_7$  versus  $C_{10}$ :  $U = 119$ ,  $P < 0.0001$ ). Titers then decayed significantly from this peak ( $M_{10}$  versus  $M_{14}$ :  $U = 151$ ,  $P = 0.0004$ ;  $C_{10}$  versus  $C_{14}$ :  $U = 191.5$ ,  $P = 0.007$ ). Despite high prevalence at all time points, we observed a higher variation in infection and dissemination for MAYV-D compared with MAYV-L (i.e.,  $M_{14}$ :  $F = 3.23$ ,  $P = 0.0026$ ;  $C_{14}$ :  $F = 4.84$ ,  $P < 0.0001$ ). Transmission trends differed slightly between the two genotypes. Unlike its infection and dissemination rates, which were high and steady, MAYV-D transmission efficiency (Figure 2A) increased over time (Table 1;  $TE_7$ : 20%;  $TE_{10}$ : 32%;  $TE_{14}$ : 48.4%), although viral titers detected in those infectious mosquitoes remained constant ( $S_{7[in]}$ :  $1.3 \times 10^3$  ffu/mL;  $S_{14[in]}$ :  $1.25 \times 10^3$  ffu/mL). Note that MAYV-D was not sampled at 21 dpi due to high mortality that differed from controls (Figure 2C, Supplemental Figure 1; 96.9%,  $\chi^2 = 43.72$ , degree of freedom [df] = 1,  $P < 0.0001$ ). For MAYV-L, virus was present in the saliva samples of about one quarter of mosquitoes at most time points (Figure 2B, Table 1;  $TE_7$ : 26.9%;

$TE_{10}$ : 23%;  $TE_{14}$ : 22.2%) but neither prevalence nor viral titers ( $S_{7[in]}$ :  $1.76 \times 10^2$  ffu/mL;  $S_{14[in]}$ :  $3.11 \times 10^2$  ffu/mL) increased significantly over time. At 21 dpi, no mosquitoes were able to transmit the virus (i.e.,  $TE_{21}$ : 0%), which can be due to viral clearance from the body. Indeed, lower titers were also detected in midgut and carcass at 21 dpi (Figure 2B; MAYV-L:  $M_{14}$  versus  $M_{21}$ :  $U = 19.5$ ,  $P < 0.0001$ ;  $C_{14}$  versus  $C_{21}$ :  $U = 8.5$ ,  $P < 0.0001$ ). However, these low titers may reflect selection bias, because mortality was also very high in MAYV-L-challenged mosquitoes at 21 dpi (Figure 2C, Supplemental Figure 1; 93.75%,  $\chi^2 = 15.59$ , df = 1,  $P < 0.0001$ ).

***An. albimanus* is not a competent vector of CHIKV or DENV-2.** Whereas MAYV was able to successfully infect and transmit through *An. albimanus* mosquitoes, this was not the case for the other assayed viruses endemic in its native range. Following challenge with CHIKV, *An. albimanus* was able to become infected at moderate levels; we detected viral presence in 33% of the mosquitoes' midguts at 7 dpi (Figure 3A;  $M_{7[in]}$ :  $2 \times 10^4$  ffu/mL), but that decreased to 10% at 10 and 14 dpi ( $M_{10[in]}$ :  $3.67 \times 10^4$  ffu/mL;  $M_{14[in]}$ :  $1.44 \times 10^4$  ffu/mL). We found that CHIKV did not efficiently escape the midgut and disseminate, likely due to a midgut escape barrier. Only two carcass samples were CHIKV positive at 7 dpi (6.7%, 2/30), and 0% of infections were disseminated at both 10 and 14 dpi (Figure 3A, Table 1). None of these carcass-infected mosquitoes progressed to infected saliva. It therefore appears that CHIKV infection, at least with the H20235 virus strain and *An. albimanus* colony strain we tested, can only be established at the midgut level and rarely disseminates. CHIKV-associated mosquito mortality was not significantly different compared with the blood-fed-only controls (Figure 3C, Supplemental Figure 1; 45.9%,  $\chi^2 = 0.82$ , df = 1,  $P = 0.365$ ).

Besides American alphaviruses, we also assessed *An. albimanus*'s vector competence for DENV-2 due to its socio-economic importance worldwide and because it is endemic in *An. albimanus*'s habitat. Consistent with previous findings in anophelines, here we report that *An. albimanus* is refractory to DENV-2 infection. Despite challenging the mosquitoes with relatively high titers ( $5 \times 10^6$  ffu/mL) and detecting positive sampling at 0 dpi (which depicts mosquito intake of an infectious bloodmeal; Figure 3B), none of the collected individuals (Figure 3B, Table 1; 0/61) from 7 to 21 dpi was found to carry infectious virus in midgut, carcass, or saliva. These results led us to question whether anophelines possess a flavivirus-specific midgut barrier, which would restrict viral replication in the midgut upon the ingestion of an infective bloodmeal, or instead they possess a body-wide infection or replication barrier against flaviviruses that prevents replication in all tissues. To test this, we injected infectious DENV-2 into the hemolymph of *An. albimanus*, thereby bypassing the hypothetical midgut barrier. To test anophelines and flaviviruses more broadly than this one species and virus, we additionally tested DENV in *An. gambiae*, *An. stephensi*, and *Anopheles quadrimaculatus* while also testing Zika virus (ZIKV) in *An. albimanus*. After 3 days of infection, none of the mosquitoes presented infectious particles in their bodies as assessed by FFA (Supplemental Figure 2), indicating that the injected virus was not able to replicate within the mosquito and that the tested anophelines are completely refractory to human flavivirus infections.

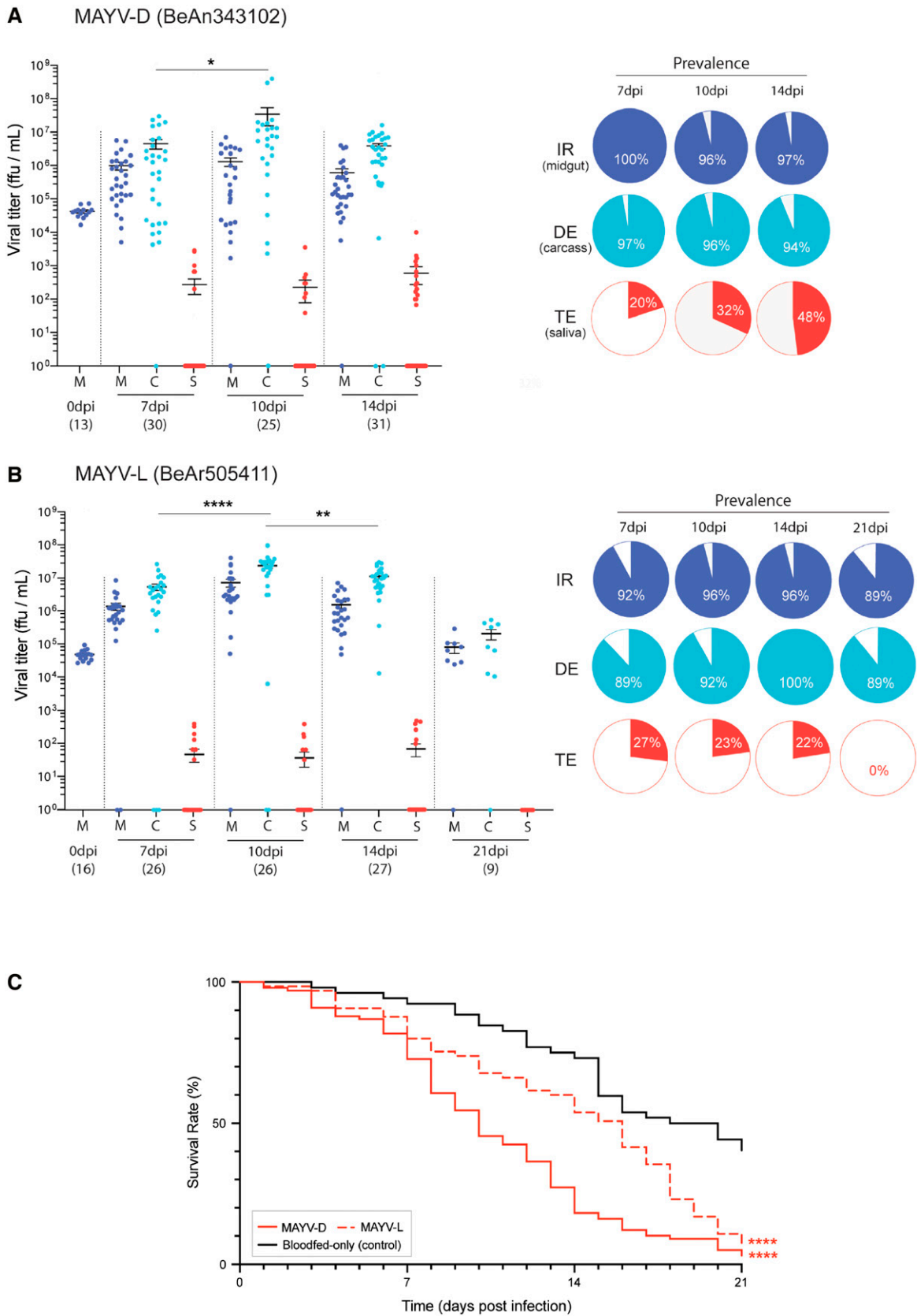


FIGURE 2. **(A and B)** Viral titer in *An. albimanus*'s midgut (M), rest of the body (carcass; C), and saliva (S) after exposure to **(A)** MAYV-D or **(B)** MAYV-L. Each dot corresponds to the titer of a single mosquito sample, with the number of collected samples (*n*) depicted below each time point. Pie charts indicate prevalence of infection (IR, dark blue), dissemination (DE, light blue), and transmission (TE, red) among the total challenged mosquitoes. **(C)** *An. albimanus*'s mortality associated with challenge and infection with MAYV strains. Statistical significance between virus-treated and blood-fed-only samples is indicated by asterisks (\**P* < 0.05; \*\**P* < 0.01; \*\*\*\**P* < 0.0001) and performed by curve comparison using a survival log-rank Mantel-Cox test.

TABLE 1  
Parameters describing infections in *An. albimanus*

Virus and collection time	% IR (x/N)	% DR (y/x)	% DE (y/N)	% TR (z/y)	% TE (z/N)
MAYV-D					
7 dpi	100 (30/30)	96.7 (29/30)	96.7 (29/30)	20.6 (6/29)	20 (6/30)
10 dpi	96 (24/25)	100 (24/24)	96 (24/25)	33.3 (8/24)	32 (8/25)
14 dpi	96.8 (30/31)	96.7 (29/30)	93.5 (29/31)	51.8 (15/29)	48.4 (15/31)
21 dpi	—	—	—	—	—
MAYV-L					
7 dpi	92.3 (24/26)	95.8 (23/24)	88.5 (23/26)	30.4 (7/23)	26.9 (7/26)
10 dpi	96.2 (25/26)	96 (24/25)	92.3 (24/26)	25 (6/24)	23 (6/26)
14 dpi	96.3 (26/27)	104 (27/26)	100 (27/27)	22.2 (6/27)	22.2 (6/27)
21 dpi	88.9 (8/9)	100 (8/8)	88.9 (8/9)	0 (0/8)	0 (0/9)
ONNV					
7 dpi	100 (26/26)	96.2 (25/26)	96.2 (25/26)	8 (2/25)	7.7 (2/26)
10 dpi	96.2 (25/26)	100 (25/25)	96.2 (25/26)	4 (1/25)	3.8 (1/26)
14 dpi	96.3 (26/27)	100 (26/26)	96.3 (26/27)	3.8 (1/26)	3.7 (1/27)
21 dpi	100 (16/16)	100 (16/16)	100 (16/16)	31.3 (5/16)	31.3 (5/16)
SINV					
7 dpi	100 (24/24)	95.8 (23/24)	95.8 (23/24)	4.3 (1/23)	4.2 (1/24)
10 dpi	100 (27/27)	92.6 (25/27)	92.6 (25/27)	4 (1/25)	3.7 (1/27)
14 dpi	94.7 (18/19)	100 (18/18)	94.7 (18/19)	11.1 (2/18)	10.5 (2/19)
21 dpi	93.8 (15/16)	100 (15/15)	93.8 (15/16)	26.7 (4/15)	25 (4/16)
CHIKV					
7 dpi	33 (10/30)	20 (2/10)	6.7 (2/30)	0 (0/2)	0 (0/30)
10 dpi	10 (3/30)	0 (0/3)	0 (0/30)	—	0 (0/30)
14 dpi	10 (3/30)	0 (0/3)	0 (0/30)	—	0 (0/30)
21 dpi	—	—	—	—	—
DENV-2					
7 dpi	0 (0/17)	—	0 (0/17)	—	0 (0/17)
10 dpi	0 (0/14)	—	0 (0/14)	—	0 (0/14)
14 dpi	0 (0/13)	—	0 (0/13)	—	0 (0/13)
21 dpi	0 (0/8)	—	0 (0/8)	—	0 (0/8)

DE = dissemination efficiency; dpi = days post infection; DR = dissemination rate; IR = infection rate; N = total number of challenged mosquitoes; TE = transmission efficiency; TR = transmission rate; x = number with virus present in midgut; y = number with virus present in carcass; z = number with virus present in saliva. IR, DR, DE, TR, and TE for all the assessed viruses are reported.

### Alphaviruses present outside the Americas can infect, disseminate, and be transmitted by *An. albimanus*.

We also asked whether *An. albimanus* may be a suitable vector of ONNV and SINV. These alphaviruses are not known to have caused outbreaks in the Americas yet, but they could emerge in currently unaffected areas due to globalization, travel, and climate change—just as ZIKV spread to new continents.<sup>26,27</sup> We found infectious ONNV virions (p5' dsONNVic) were able to both infect and disseminate from the midgut in nearly all challenged *An. albimanus* when they were fed at  $1 \times 10^7$  ffu/mL (Figure 4A, Table 1; IR: 97.9%; DR: 98.9%; DE: 96.8%), showing that the species is susceptible to the virus. Unlike the pattern observed for MAYV, the highest ONNV infection intensity in the midgut was detected at 7 dpi (Figure 4A;  $M_7 = 4 \times 10^5$  ffu/mL;  $M_7$  versus  $M_{10}$ :  $U = 90.5$ ,  $P < 0.0001$ ), which then dropped slightly and remained stable throughout the remaining time points ( $M_{10}$  versus  $M_{21}$ :  $U = 167$ ,  $P = 0.29$ ). Dissemination viral titers were similar to MAYV, peaking at 10 dpi ( $C_{10} = 2.5 \times 10^6$  ffu/mL;  $C_7$  versus  $C_{10}$ :  $U = 148.5$ ,  $P = 0.0004$ ) followed by a stable plateau ( $C_{10}$  versus  $C_{21}$ :  $U = 155.5$ ,  $P = 0.18$ ). Despite high viral prevalence in the midgut and body, transmission rates were very low early in ONNV infections (Figure 4A; TE/TR<sub>7-14</sub>: 4–7%) until an abrupt increase at 21 dpi (TE/TR<sub>21</sub>: 31.3%;  $S_{14}$  versus  $S_{21}$ :  $U = 157$ ,  $P = 0.014$ ). Our data thus show that the virus can efficiently invade the salivary glands only late in the course of infections. Mortality was not significantly different in ONNV-positive mosquitoes compared with their blood-fed-only counterparts (Figure 4C, Supplemental Figure 1; 70.4%,  $\chi^2 = 2.06$ ,  $df = 1$ ,  $P = 0.151$ ).

*An. albimanus* was also susceptible to being infected and transmitting SINV, and infection dynamics were broadly similar to those of ONNV. Almost all mosquitoes presented infected midguts (Figure 4B, Table 1; IR: 97.7%), with dissemination to the rest of their bodies also occurring at high frequencies (Figure 4B, Table 1; DR: 96.4%; DE: 94.2%). SINV titers were stable over time—only increasing in carcasses (i.e., dissemination) from 7 to 14 dpi ( $U = 115$ ,  $P = 0.005$ ). This may indicate a slower replication rate within the mosquito tissues that delays and reduces its vectorial capacity. Early in the course of infections, few saliva samples presented infectious SINV ( $S_{7-10}$ : 4%;  $S_{14}$ : 11%) and those that did had low titers. Similar to ONNV, viral loads in saliva rose significantly at the latest time point (TE<sub>21</sub>: 25%;  $S_{21in}$ :  $3.7 \times 10^2$  pfu/mL), again representing a slower transition from midguts to the salivary glands.

### DISCUSSION

Here, we demonstrate that *An. albimanus*, the most common *Anopheles* mosquito in Mesoamerica and the Caribbean,<sup>8,16,28</sup> is a competent laboratory vector for a broad range of alphaviruses but refractory to flavivirus infection. Although arboviral spread through anophelines has received little research attention, we found this species was readily infected and transmitted three of five tested viruses—suggesting that it (and possibly other anophelines) may be susceptible to infection with a substantial number of viruses.<sup>3</sup> Although the tested alphaviruses (except CHIKV) were able to establish disseminated infections and be transmitted by

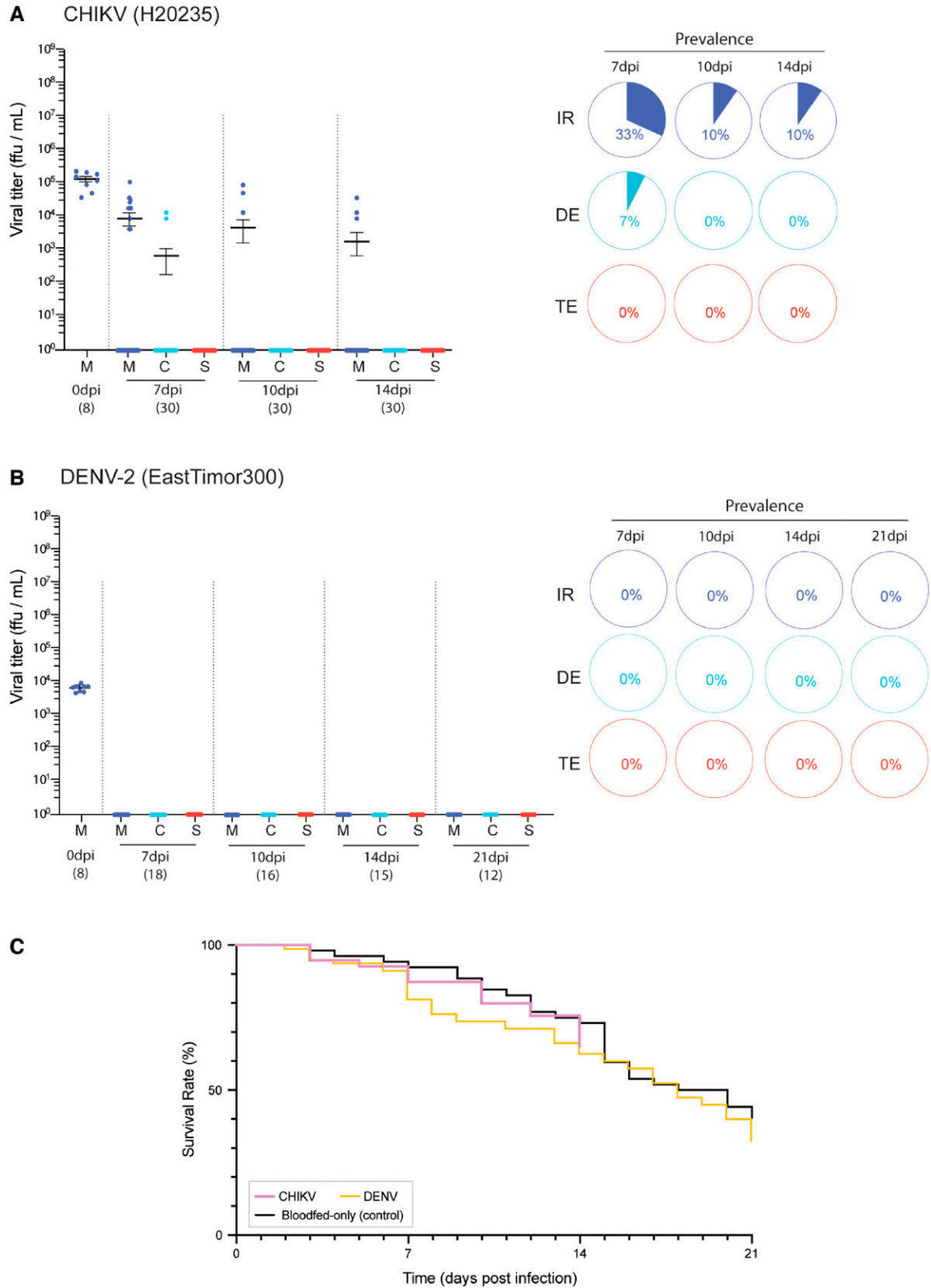
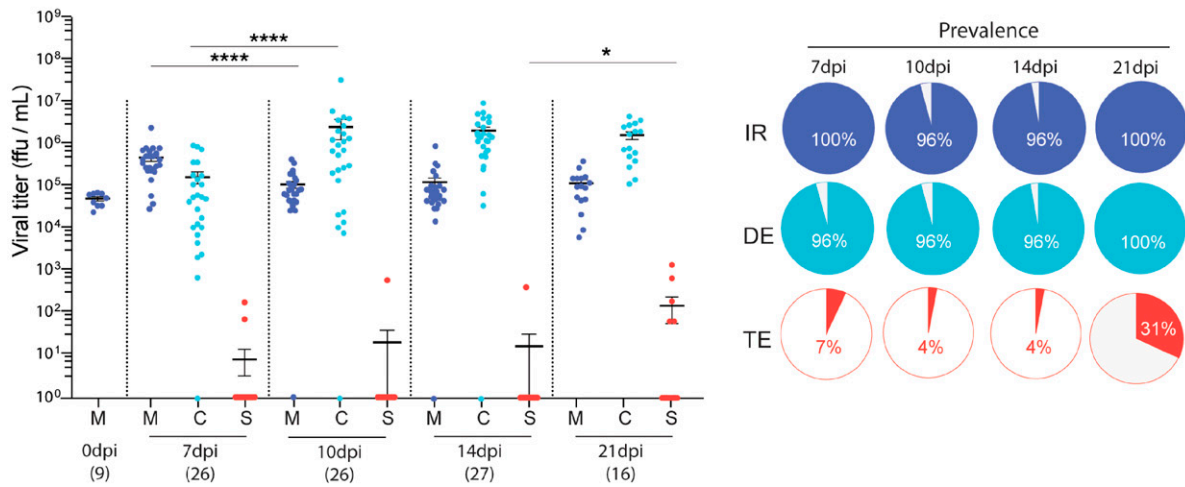
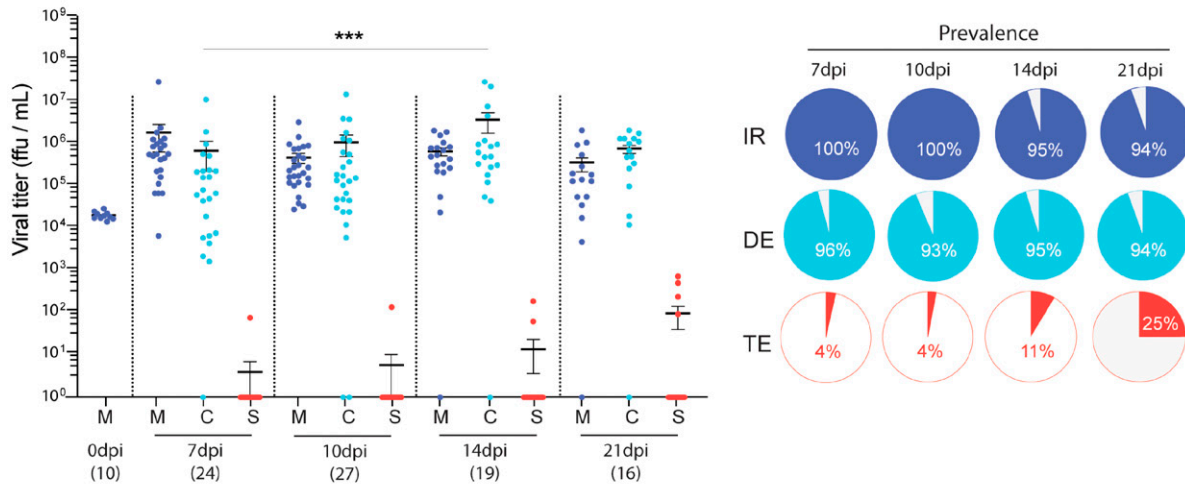


FIGURE 3. (A and B) Viral titer in *An. albimanus*'s midgut (M), rest of the body (carcass; C), and saliva (S) after exposure to (A) CHIKV or (B) DENV-2. Each dot corresponds to the titer of a single mosquito sample, with number of collected samples (*n*) depicted under each time point. Pie charts indicate prevalence of infection (IR, dark blue), dissemination (DE, light blue), and transmission (TE, red) among the total challenged mosquitoes. (C) *An. albimanus*'s mortality associated with challenge and infection with CHIKV and DENV-2. Statistical significance between virus-treated and blood-fed-only samples was performed by curve comparison using a survival log-rank Mantel-Cox test.

**A** ONNV (SG-650)



**B** SINV (MRE16)



**C**

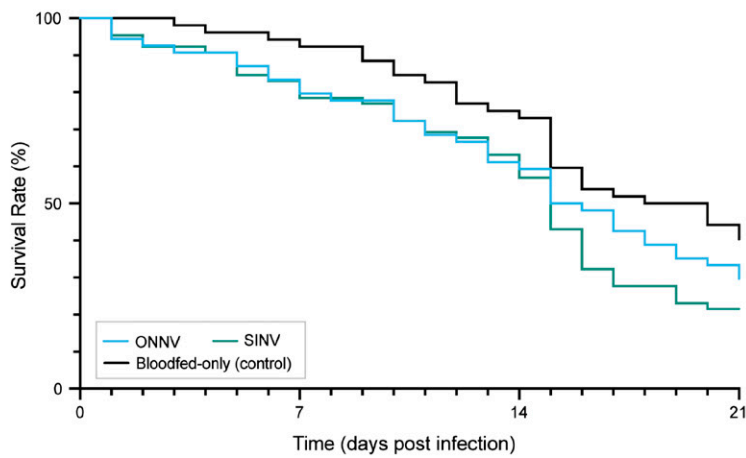


FIGURE 4. **(A and B)** Viral titer in *An. albimanus*'s midgut (M), rest of the body (carcass; C), and saliva (S) after exposure to **(A)** ONNV or **(B)** SINV. Each dot corresponds to the titer of a single mosquito sample, with number of collected samples (*n*) depicted under each time point. Pie charts indicate prevalence of infection (IR, dark blue), dissemination (DE, light blue), and transmission (TE, red) among the total challenged mosquitoes. **(C)** *An. albimanus*'s mortality associated with challenge and infection with ONNV and SINV strains. Statistical significance between virus-treated and blood-fed-only samples is indicated by an asterisk (\**P* < 0.05; \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001) and performed by curve comparison using a survival log-rank Mantel-Cox test.

this species, they presented different patterns of infection and transmission.

Notably, an alphavirus that is endemic to the Americas (MAYV)—and that therefore has the potential to infect *An. albimanus* in the wild—had the highest transmission efficiency of the tested viruses. Both MAYV genotypes (-D and -L; Figure 2A and B, respectively) are able to escape the mosquito's tissue barriers,<sup>29</sup> replicating freely within the body of the mosquito and reaching the salivary gland lumen. We found MAYV infections progressed the most rapidly, as indicated by higher titers in the midguts and body, as well as earlier and greater prevalence in saliva. Saliva samples tested positive for MAYV at a substantial rate (20–48%) at all surveyed time points except 21 dpi, when most mosquitoes that potentially carried high viremia may have suffered from MAYV-associated mortality (Figure 2C, Supplemental Figure 1). This increased mortality likely affects their vectorial capacity in the wild, because mosquitoes may die prematurely, or because they may be infectious but not vigorous enough to seek out and bite hosts. However, it remains unclear whether (and how often) *An. albimanus* becomes infected with MAYV in the wild.

Our data also suggest there may be differences in how the two MAYV subgroups infect the tested colony of *An. albimanus*. For example, we detected higher variance in MAYV-D infection titers compared with those of MAYV-L infections (Figure 2A versus Figure 2B). Although both show a short extrinsic incubation period<sup>30</sup> that represents heightened vectorial capacity, MAYV-D's transmission increased over time whereas MAYV-L remained consistent at around 25%, although this pattern was not statistically significant. Similar variation between MAYV genotypes in infection was previously observed in *An. gambiae*.<sup>20</sup> These differences suggest there may be interesting phenotypical variation in infection that could be explored in future studies.

To date, few MAYV outbreaks have been detected in humans, with most infections occurring in the Amazon Basin.<sup>31</sup> However, recent studies indicate that MAYV's true incidence may be underestimated<sup>32</sup> because its symptoms resemble those of other pathogens in the area (DENV, CHIKV). Epidemiological concern is rising due to MAYV's ability to colonize urban areas and its potential to be transmitted through lesser-known vectors of arboviruses in Central and South America.<sup>31,33</sup> With the geographical range expansion of *An. albimanus* into lower latitudes of South America as well as northward into the United States, coupled with the high viral susceptibility and transmission observed in a laboratory colony, there is vectorial potential for this species to drive such outbreaks.

Not all alphaviruses endemic to the Americas were successful in infecting *An. albimanus*. Most notably, CHIKV had poor prevalence in challenged mosquitoes. Differences in transmission capacity can be explained by dynamic and complex virus–vector interactions that affect the vectorial capacity of the mosquito species,<sup>34</sup> and which may trace back to variation in host-to-pathogen genotype interactions (GxG). GxG interactions govern outcomes in many systems, including arboviral infections<sup>35</sup> and symbiosis<sup>36</sup> in insects. In some cases,<sup>26,27</sup> the combination of a specific insect genotype and a specific pathogen genotype strongly shapes infection status and progression. Although *Anopheles* are not typically vectors for CHIKV,<sup>37</sup> some species including *An. stephensi* can harbor and transmit it.<sup>24</sup> In these rare cases,

GxG interactions may be key to the ability of the mosquito to transmit the virus. Consistent with what has been observed for most anophelines, *An. albimanus* were able to harbor the infection following a viral bloodmeal but were refractory to dissemination and transmission of CHIKV (Figure 3A). One hypothesis is that *An. albimanus* has coevolved with this strain of CHIKV (collected on the Caribbean island of St. Martin) and the GxG interaction between mosquito and virus is not ideal.<sup>35,38</sup> Of course, our findings do not exclude the possibility that CHIKV may be transmitted through *An. albimanus* mosquitoes in some circumstances, because these results were obtained using a single laboratory colony. It remains possible that wild strains from other locations may present a more suitable combination for the virus to transmit—by differing either in their nuclear genotype<sup>35</sup> or microbiome composition.<sup>39</sup>

We found that *An. albimanus* can also carry and transmit the non-American alphaviruses ONNV and SINV (Figure 4A and B). These two viruses affect different geographical and climate regions and are primarily transmitted by other mosquito species.<sup>40,41</sup> ONNV is closely related to CHIKV and MAYV (all belong to the Semliki Forest antigenic complex) and causes outbreaks in humans in Central Africa. In contrast, SINV is a distant relative. It belongs to the Western equine encephalitis complex and is the causal agent of Pogosta disease, mostly in northern Europe<sup>42</sup> and southern Africa,<sup>43</sup> with sporadic cases detected in Australia.<sup>44</sup> Despite their biological differences, both ONNV and SINV are able to infect *An. albimanus*, albeit with slower transmission kinetics compared with MAYV. More specifically, although infected early at the midgut level, very few ONNV- or SINV-challenged mosquitoes were saliva positive at the earliest time point tested (7 dpi). Rather, their highest transmission was detected at 21 dpi for both viruses. This longer time of incubation (and the mortality observed over time) suggests that, even though viral transmission rates are somewhat elevated at that time, mosquito survival is decreasing rapidly due to aging and infection status and should not necessarily pose a higher risk of transmission epidemiologically. Although neither ONNV nor SINV has been detected in the Americas yet, globalization and human travel<sup>45</sup> pose a risk for these viruses to spread there, especially ONNV, whose primary vectors in Africa include the anopheline species *An. gambiae* and *An. funestus*<sup>46,47</sup> whereas SINV is mainly transmitted by culicine species.<sup>48</sup>

We found multiple anopheline species were refractory to infection with the tested flaviviruses DENV-2 and ZIKV, even when virus was injected directly into the hemolymph. The inability to harbor these flaviviruses could trace back to a variety of different mechanisms, for example, lack of a particular replication machinery component<sup>49</sup> or absence of a specific cellular receptor or factor required for infection success.<sup>50–52</sup> However, there are reports of insect-specific flavivirus infections in anophelines,<sup>53,54</sup> raising questions about the underlying mechanism and host restriction features of different flaviviruses, and whether some specific human flavivirus infections do occur in anophelines given permissive GxG interactions.

In short, this study tested the capacity of *An. albimanus* to be an important vector of alphaviruses in the Americas. Our data show that, although *An. albimanus* is unlikely to drive CHIKV and flavivirus infections, other alphaviruses (especially MAYV) can infect and be transmitted through this mosquito species very efficiently. Our results highlight both the



importance for *Anopheles* mosquitoes to be recognized as potential vectors of arboviruses as well as the knowledge gaps that still need to be filled on the genus's vectorial capacities worldwide.

## MATERIALS AND METHODS

**Mosquitoes.** The *An. albimanus* mosquito colony (STECLA strain, MRA-126) was kept and reared at the Millennium Science Complex insectary (Center for Infectious Disease Dynamics, The Pennsylvania State University) at a continuous  $27 \pm 1^\circ\text{C}$ , 85% relative humidity, 12:12-hour light:dark cycle. Larvae were fed koi pellets (Tetra Pond Koi Vibrance; Tetra, Melle, Germany) from hatching to pupation. Adult mosquitoes were reared in  $30 \times 30 \times 30$ -cm metal cages and provided 10% sucrose solution ad libitum, as well as fed weekly with anonymous human blood (Biological Specialty, Colmar, PA) for reproduction and colony maintenance using a membrane feeder.

**Cells.** African green monkey kidney cells (Vero; CCL-81) and *Aedes albopictus* larval cells (C6/36; CCL-126) (ATCC, Manassas, VA) were cultured in complete media consisting of Dulbecco's modified Eagle's medium or RPMI-1640, respectively, complemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin—all reagents purchased from Gibco, Thermo Fisher Scientific (Waltham, MA). For each passage, cells were detached by trypsinization (0.25% trypsin; Corning Inc., Corning, NY) and diluted in fresh complete media or plated for experiments.

**Viruses.** A total of six different viruses was used for experimental infections (Figure 1A). Two strains of MAYV were used: BeAn343102 (BEI Resources, Manassas, VA) is a genotype D strain (MAYV-D) isolated in May 1978 from a monkey in Para, Brazil, and BeAr505411 (BEI Resources) is a genotype L strain (MAYV-L) also isolated in Para, Brazil in March 1991 from *Haemagogus janthinomys* mosquitoes. The full-length ONNV and SINV infectious clones (p5'dsONNVic and p5'dsMRE16ic) derive from the Uganda SG-650 strain of ONNV<sup>55</sup> and wild-type MRE16 strain of SINV isolated from Malaysia,<sup>56</sup> respectively. Both infectious clones were obtained on filter paper and transfected, and virions were collected prior to passaging in Vero cells for the experiments. The DENV-2 ET300 strain was isolated from a human patient in Timor-Leste in 2000 (GenBank accession number EF440433.1). Lastly, the CHIKV H20235 strain (NR-49901; BEI Resources) was isolated from a human in St. Martin in 2013. All work with this strain (from cell culture to mosquito infections) was performed in the Eva J. Pell Laboratory for Advanced Biological Research BSL-3 facility at The Pennsylvania State University.

All alphaviruses were passed in African green monkey kidney (Vero) cells at  $37^\circ\text{C}$  in a humidified 5%  $\text{CO}_2$  incubator, whereas DENV-2 was passed in *Ae. albopictus* RNA interference-deficient C6/36 cells at  $28^\circ\text{C}$ . Viruses were allowed to infect cells at a multiplicity of infection of 0.1 for 1 hour and then removed and replaced with media containing 2% FBS. Virus-infected supernatant was aliquoted at different time points (typically 24 hpi for alphaviruses and 7 dpi for DENV-2) and stored at  $-80^\circ\text{C}$  until further titration or use for mosquito infections. Viral stock titers were obtained using FFAs (ffu/mL) or PFAs (pfu/mL), as described below.

**Vector competence assays.** To determine the vectorial competence of *An. albimanus*, adult females were orally challenged with an infected bloodmeal containing a high-titer dose of one of the five togaviruses or DENV-2. Specifically, 6- to 8-day-old non-blood-fed females were allowed to feed on human blood for 1 hour through a synthetic membrane at the bottom of a glass feeder jacketed with  $37^\circ\text{C}$  water and containing either  $1 \times 10^7$  ffu/mL (alphaviruses) or  $5 \times 10^6$  ffu/mL (DENV-2) of the stocks obtained above (Figure 1C, gray boxes). Fully engorged mosquitoes were sorted from non-fed ones and split evenly in cups for each collection time point. A small subset of mosquitoes was collected at 0 dpi to confirm that the viral intake was infectious and similar across samples.

Infection rate, dissemination and transmission rates (DR and TR), as well as dissemination and transmission efficiency (DE and TE) were assessed at 7, 10, 14, and 21 dpi. IR was measured as the rate of mosquitoes with infected midguts among the total number of mosquitoes. DR and DE were measured as the rate of mosquitoes with infected carcasses among the mosquitoes with infected midguts or over the total assessed samples, respectively. TR and TE were measured as the rate of mosquitoes with infectious saliva among the positive bodies or the total number of assessed mosquitoes, respectively.

At all time points, mosquitoes were anesthetized using triethylamine (Sigma, St. Louis, MO) before individual forced salivation. Saliva was collected by placing the female's proboscis into a pipette tip containing 20  $\mu\text{L}$  of a 50% sucrose, 50% FBS solution, as previously described,<sup>57</sup> for 30 minutes. Solution was then released into a tube filled with 100  $\mu\text{L}$  of mosquito diluent (20% heat-inactivated FBS, 50  $\mu\text{g}/\text{mL}$  penicillin/streptomycin, 50  $\mu\text{g}/\text{mL}$  gentamicin, and 2.5  $\mu\text{g}/\text{mL}$  fungizone in Dulbecco's phosphate-buffered saline [PBS]) and placed on ice. Each female's midgut was dissected and placed in a 2-mL tube containing 300  $\mu\text{L}$  of mosquito diluent. The rest of the body (carcass) was also collected in an identical tube. Tissue samples were homogenized at 30 Hz for 2 min using a Tissue-Lyser II (QIAGEN, Hilden, Germany). All samples were stored at  $-80^\circ\text{C}$  until viral testing.

**Intrathoracic injections.** *Anopheles gambiae*, *An. stephensi*, *An. quadrimaculatus*, and *An. albimanus* females were briefly anesthetized on a chill block (BioQuip Products, Compton, CA) cooled to  $4^\circ\text{C}$  and DENV-2 and ZIKV stocks were injected intrathoracically under a microscope using a pulled glass capillary with a manual microinjector (Nanoject II, Drummond Scientific, Broomall, PA) to ensure uniformity of dosage. Sixty-nine microliters of diluted virus stock ( $\sim 70$  DENV-2 pfu) were delivered intrathoracically into each female. After injection, mosquitoes were maintained under standard housing conditions of  $27^\circ\text{C}$  with 80% relative humidity and 12:12-hour light:dark cycle and fed 10% sucrose solution ad libitum.

**Focus-forming assay.** The presence of infectious particles of all viruses except SINV in saliva, midguts, and carcasses was tested by FFAs in Vero (alphaviruses) or C6/36 (DENV-2) cells. Cells were counted using a hemacytometer (Hausser Scientific, Horsham, PA) and plated in complete media the day before infection to achieve 80–90% confluency (Vero:  $3 \times 10^4$  cells per well; C6/36:  $2 \times 10^5$  cells per well) in 96-well plates. The following day, media were removed from all wells, and cells were incubated for 1 hour with 30  $\mu\text{L}$  of 10-fold dilutions ( $10^{-1}$  to  $10^{-4}$ ) of each

homogenized tissue sample in FBS-free media. Saliva samples were not diluted, due to their lower titers. Viral media were removed from the wells after 1 hour, replaced with 100  $\mu$ L of overlay (final 0.8% methylcellulose [or CMC] in complete media), and incubated at 37°C for 24 hours or 28°C for 3 days, depending on the cell culture used. Cells were then fixed using 4% paraformaldehyde in PBS (Sigma-Aldrich) for 20 min and permeabilized with 0.2% Triton-X in PBS for another 20 minutes. Samples were washed two or three times with cold 1 $\times$  PBS after both fixation and permeabilization steps. Viral antigens in infected cells were labeled overnight using mouse monoclonal anti-CHIKV E2 envelope glycoprotein clone CHK-48 (for all alphaviruses except SINV;  $\alpha$ -CHK-48, BEI Resources) or mouse monoclonal anti-flavivirus clone D1-4G2-4-15 (for DENV-2; BEI Resources) diluted 1:500 in PBS. The next day, cells were again washed thoroughly with cold PBS to remove unbound primary antibody. Bound primary antibody was then labeled for 1 hour at room temperature using an Alexa 488 goat anti-mouse IgG secondary antibody (Invitrogen, OR Waltham, MA) at a 1:750 dilution in PBS, which was then rinsed off with reverse osmosis water before evaluation by fluorescence microscopy. Green fluorescence was observed using a fluorescein isothiocyanate filter on an Olympus BX41 microscope with a UPlanFI 4 $\times$  objective. Foci were counted by eye in the appropriate dilution (10–100 foci) and viral titers were backcalculated to ffu/mL.

**Plaque-forming assay.** The  $\alpha$ -CHK-48 antibody used in FFAs does not cross-react with SINV, which is evolutionarily the most distantly related to CHIKV.<sup>58</sup> Thus, we elected to assess mosquito SINV infections by traditional PFAs, which are comparable to FFAs because both detect the presence of infectious viral particles in a sample using a cell-based method.

Mosquito samples were tested for SINV infectious particles by plaque assay on Vero cells with minimal modifications.<sup>59</sup> The day before infection, cells were counted as described above and plated ( $5 \times 10^6$  cells per well) in six-well plates. For saliva infections, media were removed and replaced for 100  $\mu$ L of undiluted sample. For midguts or carcasses, 10-fold dilutions using FBS-free media were performed and 180  $\mu$ L of each dilution (in most instances,  $10^{-2}$  to  $10^{-4}$ ) was used for cellular infection. Inoculated plates were placed in a 37°C incubator with 5% CO<sub>2</sub> for 1 hour for viral entry to occur. Then, virus-containing media were removed and replaced with 1.5 mL of an agar overlay (equal parts complete media and 1.2% agarose) and placed back into the incubator. After 2 days, 1.5 mL of a second agar overlay (identical to the first agar overlay but containing a 1% final concentration of neutral red [Amresco, Solon, OH] to allow for cellular staining) was added to each well and plates were incubated. The following day, agar discs were removed, and samples were treated with 4% formaldehyde to inactivate any remaining virus. Each stained well was rinsed thoroughly with water and set aside to dry well. Wells that produced 10–100 plaques were used to ensure accurate counts, and viral titers for mosquito saliva, midguts, and carcasses were calculated in pfu/mL. When samples produced too many plaques to count, additional plaque assays were performed with extra 10-fold dilutions.

**Statistical analysis and figure generation.** Differences in viral titer between midgut, carcass, and saliva samples were assessed by two-tailed nonparametric Mann-Whitney *U* tests

due to the nonnormality of the samples. In a few cases where samples were normally distributed, we used a parametric Welch's *t* test instead. Survival data for each virus were compared pairwise to a blood-fed-only control sample using a log-rank Mantel-Cox test. All *P* values that were below 0.05 (*P* < 0.05) were considered significant. All data were initially plotted and analyzed using Prism software version 9.2.0 (283) (Graph-Pad, San Diego, CA). Final figures were assembled using Adobe Illustrator 2021 (25.4.1; Adobe, San Jose, CA).

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**Authors' addresses:** Gerard Terradas, Mario Novelo, Hillery Metz, and Jason Rasgon, Department of Entomology, The Pennsylvania State University, University Park, PA, Center for Infectious Disease Dynamics, The Pennsylvania State University, University Park, PA, and Huck Institutes of the Life Sciences, The Pennsylvania State University, University Park, PA, E-mails: [gkt5113@psu.edu](mailto:gkt5113@psu.edu), [mnovelo19@gmail.com](mailto:mnovelo19@gmail.com), [hillerymetz@gmail.com](mailto:hillerymetz@gmail.com), and [jlr54@psu.edu](mailto:jlr54@psu.edu). Marco Brustolin, Department of Entomology, The Pennsylvania State University, University Park, PA, Center for Infectious Disease Dynamics, The Pennsylvania State University, University Park, PA, and Huck Institutes of the Life Sciences, The Pennsylvania State University, University Park, PA, and Instituut voor Tropische Geneeskunde, Antwerp, Belgium, E-mail: [marco.brustolin85@gmail.com](mailto:marco.brustolin85@gmail.com).

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