Immune-related transcripts, microbiota and vector competence differ in dengue-infected geographically distinct Aedes aegypti populations

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Abstract

Background

Vector competence in *Aedes aegypti* is influenced by various factors and, by recognizing what affects virus and mosquito interactions, crucial new control methods can be developed.

Methods

Here we used three geographically distinct *Ae. aegypti* populations and compared their susceptibility to infection by dengue virus (DENV). To identify the population differences, we screened immune-related genes and microbiota which might contribute to the uniqueness in competency.

Results

Based on the results from the DENV competence study, we assigned a refractory population (Vilas do Atlântico), a susceptible population (Vero), and a susceptible but low transmission population (California). The immune-related transcripts were highly expressed in the California population but not in the refractory population. However, the Rel-1 gene upregulated after blood-feeding in the Vilas do Atlântico population suggesting involvement in non-viral responses, such as response to microbiota. The bacteria, fungi, and flaviviruses screening showed differences between populations and could be one of the factors that interfere with the vector competence.

Conclusions

All in all, we revealed the potential factors that might impact the virus and mosquito interaction, moreover, influencing the *Aedes aegypti* refractory phenotype.

Background

Dengue virus (DENV) imperils people living in tropical and subtropical areas and every year 50–100 million individuals suffer symptomatic infections [1, 2]. The models of DENV-infection risk predict greater annual exposure in the near future [3]. DENV is a mosquito-borne disease transmitted by vector species in the genus *Aedes*, especially the principal vector *Aedes aegypti* [4]. Since globalization, *Ae. aegypti* spread to other areas from trading or traveling. Nowadays, these populations can be found in the Americas, Europe, Africa, Asia, and Oceania and because of global climate change, expansion of their distribution area is expected [5].

For mosquito-borne viruses, vector competence is defined as the ability of a mosquito to serve as a disease vector and encompasses midgut infection, dissemination to other tissues, and transmission of
the virus through saliva [6]. For a mosquito bite to become infectious, the virus must pass several physiological barriers including the midgut infection barrier (MIB), midgut escape barrier (MEB), salivary gland infection barrier (SGIB), and salivary gland escape barrier (SGEB) [7]. The interval between acquisition of virus by a vector and the vector becoming capable of transmission is called the extrinsic incubation period (EIP) [8]. The EIP is an important parameter that influences vectorial capacity and has been shown to vary among populations infected with flaviviruses, such as yellow fever virus (YFV), DENV, and Zika virus (ZIKV), as well as with alphaviruses, such as chikungunya virus (CHIKV) [9].

The mosquito innate immune response is activated once pathogens are recognized and can influence vector competence [6]. The RNA interference (RNAi) pathway is considered the main antiviral pathway in mosquitoes and is involved in the degradation of viral RNA [10–13]. Antiviral activity is also regulated via several innate immune pathways [14], including the Toll pathway [15, 16], the immune deficiency (IMD) pathway [17, 18], and the Janus Kinase/signal transducer and activator of transcription (JAK/STAT) pathway [19, 20]. These three major pathways involve pattern recognition followed by signal transduction to trigger the expression of downstream antimicrobial peptides or other immune factors. Some non-traditional immune pathways might also participate in the antiviral response such as apoptosis [21, 22] and autophagy [22, 23].

Mosquito microbiota including bacteria, fungi, and insect-specific viruses (ISVs) can influence a range of host phenotypes, including vector competence [24–29]. Bacteria in Ae. aegypti were shown to influence DENV infection [16, 30–32]. Moreover, both insect-specific viruses and fungi can also impact vector competence and alter virus infection dynamics within the mosquito [27, 33–35]. The microbiota is diverse within and between mosquito populations [24] and varies in mosquito strains that are differentially susceptible to viruses [36].

In this study, three geographically distinct Ae. aegypti populations were compared for their susceptibility to infection and ability to transmit DENV-2. To identify potential factors responsible for differences in vector competence, we focused on each of the major immune pathways, selecting key genes in three categories to represent pathways. These included (i) transcription factors, such as Rel-1A (Toll), Rel-1B (Toll), Rel-2 (IMD); (ii) receptors, such as Dome (JAK/STAT); or (iii) effectors, such as ATG5 (autophagy), Ago-2 (RNAi), Dronc (apoptosis) and IAP-1 (apoptosis). We then characterized their transcript levels after DENV-2 infection during the first three days of the extrinsic incubation period and compared expression profiles among populations. To examine additional factors likely underlying variation in vector competence, we screened bacterial, fungal, and viral components of the microbiota by PCR. We conclude by discussing the potential connection of variation in the microbiota and innate immunity to population-level differences in DENV infection dynamics within the mosquito.

Methods

Mosquitoes
Eggs of *Ae. aegypti* were collected in the cities of Salvador (Vilas do Atlântico area) in Bahia, Brazil, using standard oviposition traps from 2015 to 2017. Mosquito eggs from California (collected in 2016) and Vero populations (collected in 2015 from Vero Beach, Florida) were the two North American populations used in the study. *Aedes aegypti* were reared at 28°C and 60–80% relative humidity in a climate-controlled room with a light: dark cycle of 14:10 hours. After hatching, larvae were separated into pans at an approximate density of 200 larvae per pan and provided with 3 ml of larval food (5g brewer’s yeast and 5g liver powder mixed with 1 L water) daily. Adults were provided 20% sucrose solution-soaked cotton rolls ad libitum. Mosquito colonies were maintained by feeding female mosquitoes on blood from chickens following approved standard protocols (IACUC protocol 201807682) [37, 38]. The Vilas do Atlântico F4, California F7 and Vero F8 generations were used in the study.

**Infection of** *Ae. aegypti*

Four to six-day-old *Ae. aegypti* females were placed into two groups (control and infected). The control group contained 150 females from each population fed on defibrinated bovine blood (Hemostat, Dixon, CA, USA) without virus. The infection group with 250 females was fed on defibrinated bovine blood containing DENV serotype 2 (DENV-2) (New Guinea C strain, GenBank accession # KM204118). The virus was inoculated into African green monkey (Vero) cells at a multiplicity of infection of 0.1 viruses per cell and incubated at 37°C with 5% CO2 for 5 days. One day before infection, female mosquitoes were transferred to 16 oz paperboard cartons (WebstaurantStore, Lancaster, PA, USA) and placed in an incubator under the same conditions as the climate-controlled room. Sugar water was removed 24 hours before blood feeding. The defibrinated bovine blood (Hemostat, Dixon, CA, USA) mixed with supernatant from fresh virus culture or medium from 5 day-old culture of Vero cells without virus was supplied to mosquitoes using an artificial feeding apparatus (Hemotek, Lancashire, United Kingdom). The ratio of blood to medium with or without virus was two to one. The feeding duration was one hour. After feeding, fully blood-engorged mosquitoes were collected into cartons and provided with 20% sucrose solution ad libitum. Freshly fed, fully blood-engorged mosquitoes in each population and a blood sample from the assay were collected immediately following feeding for determining virus titer. Female mosquitoes were collected from the control and infected groups at each time point (24, 48, 72 hours) in four replicates of five mosquitoes per replicate and placed at -80°C. Four infected mosquitoes were also collected at 24, 48 and 72 hours to measure the virus titer. On day 10, the remaining mosquitoes in each population were collected and separated into bodies and legs. Saliva was collected after legs but before bodies using the capillary method following well-established protocols [39]. All samples were placed at -80°C for subsequent RNA extraction.

**RNA extraction**

TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) was used to extract RNA from all mosquito samples as described [39]. RNA from 10 dpi mosquito bodies, legs, and saliva was used to estimate virus titers and determine infection rates (number with viral RNA detected in the body/ number of blood-engorged mosquitoes), dissemination rates (number with viral RNA detected in the legs/ number with viral RNA detected in the body), and transmission rates (number with viral RNA detected in the saliva/ number with
viral RNA detected in the legs), respectively. Separated body and leg samples were placed in 1.5ml Eppendorf tubes with 0.2ml TRIzol and ten 2mm glass beads (Fisher Scientific, Waltham, MA, USA) and homogenized using a bullet blender tissue homogenizer (Next Advance, Troy, NY, USA). After homogenization, standard RNA extraction was completed following well-established protocols [38, 40]. Briefly, 0.3ml TRIzol was added to the homogenized tissue solution, and after incubation for 10 minutes, 0.1 ml of chloroform was added. After a 15 min incubation and centrifugation the aqueous phase was collected and mixed with 0.25ml isopropanol. Following centrifugation for 10 minutes, supernatant was discarded, and the pel let was washed in 70% ethanol and resuspended into DEPC treated water. Saliva samples preserved in 0.1 ml phosphate-buffered saline (PBS, pH 7.4) solution were extracted following the RNA extraction protocol described above. Whole mosquito bodies were collected individually and in pools of five for RNA extraction after 1, 2 and 3 dpi following the same protocol described above. All RNA samples were stored at −80°C.

**Reverse transcription and real-time polymerase chain reaction**

All RNA samples were quantified by Nanodrop 2000 spectrophotometry (Thermo Fisher Scientific, Waltham, MA, USA). RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA) to degrade any DNA carried over from RNA extraction. For the samples examining gene expression, Enhanced Avian Reverse Transcriptase (Sigma- Aldrich, St. Louis, MO, USA) was used for reverse transcription, and oligo dT primer was used to generate cDNA following standard protocols. Gene expression was characterized on a Bio-Rad CFX96™ Real-Time PCR machine using the SsoAdvanced SYBR Green Supermix kit (Bio-Rad, Hercules, CA, USA), specific primer sets designed to amplify target genes in the mosquito innate immune pathways [23, 38] with 100 ng of cDNA per sample (Table S1). Ae. aegypti ribosomal protein S7 gene (GenBank Accession # AY380336) was used as a control for standardizing and the 2^-delta-delta Ct method was applied for calculating relative gene expression values [39]. All the samples were duplexed and followed well-established protocols [40, 41]. In the virus titer quantification experiment, the virus genome equivalents were estimated by quantitative RT-PCR standardized with plaque assay as described before [42]. In brief, RNA samples were quantified and were detected on a Bio-Rad CFX96™ Real-Time PCR machine using the iTaq Universal One-Step RT-qPCR Kits (Bio-Rad, Hercules, CA, USA). The values were applied into the regression line built from the plaque assay and the log10 plaque-forming unit equivalents of DENV-2/ml (log PFUe/ml) were estimated.

In the flavivirus ISV screening study, 100 ng DNase treated uninfected mosquito RNA was combined with flavivirus universal primers at the concentration used in a previous study [43] and reagents from the iTaq Universal One-Step RT-qPCR Kit (Bio-Rad, Hercules, CA, USA) and placed into a Bio-Rad CFX96™ Real-Time PCR machine. After the reaction, positive samples were analyzed by gel electrophoresis on a 1% agarose gel. Distinct bands of the expected length were excised, and PCR amplicons were extracted using the GenElute Gel Extraction Kit (Sigma- Aldrich, St. Louis, MO, USA) and a second PCR reaction was conducted with DreamTaq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) using the same flavivirus primer set to generate a sufficient amount of DNA for cloning. PCR conditions were one cycle of 95°C for 3 min, then 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by one
cycle of 72°C for 5 min. The PCR products were cleaned up using the GenElute Gel Extraction Kit (Sigma-Aldrich, St. Louis, MO, USA) as described and placed at -20°C for cloning.

## DNA extraction and cloning of microbial PCR amplicons

To examine variation among populations for bacterial/fungal microbiota, three pools of three blood fed female mosquitoes per population were collected at 2 days post-feeding. DNA was extracted from each pool using the E.Z.N.A. Tissue DNA kit (Omega Bio-Tek, Norcross, GA, USA) and stored at -80°C until use. The DNA samples were used as templates for PCRs targeting bacterial 16S rRNA [44] and fungal 18S rRNA genes [45]. These PCR products, as well as those from the flavivirus PCR described above, were cloned (CloneJET PCR Cloning Kit, Thermo Fisher Scientific, Waltham, MA, USA) into NEB 5-alpha E. coli (New England Biolabs, Ipswich, MA, USA) and grown on selective LB agar plates with Carbenicillin (100 µg/ml). All the colonies from each sample were picked for both 16S and 18S to screen for sequence variation using restriction fragment length polymorphism analysis (RFLPs) [46]. Briefly, plasmid-specific primers (pJET1.2 Forward and Reverse) were used to amplify DNA inserts, after which the amplicons were digested with restriction enzymes Alul and Hhal (New England Biolabs, Ipswich, MA, USA) for fungi and HpalI, Clal, and HinP1I (New England Biolabs, Ipswich, MA, USA) for bacteria and electrophoresed on 1% agarose gels. Differentiating amplicon variants or RFLP patterns of 16S rRNA bacterial and 18S rRNA fungal PCR products were obtained. Colonies that had unique banding patterns were grown overnight in LB liquid media with Carbenicillin (100µg/ml). Plasmid DNA was purified using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, MA, USA), spectrophotometrically quantified and submitted for Sanger sequencing (Eurofins Genomics, Louisville, KY, USA). Flavivirus colonies were selected randomly from each population and were not subjected to RFLP. The bacterial / fungal and flavivirus OTUs (operational taxonomic units) were then identified by BLAST via the NCBI database.

## Statistical analysis

Fisher's exact test was used to compare rates of feeding, infection, dissemination, and transmission in the vector competence study. Virus titer was analyzed from freshly fed and 10 dpi mosquitoes with Kruskal-Wallis test and Dunn's test for multiple comparisons. To compare Toll pathway gene transcript levels after blood-feeding and virus titer in the first three days of the incubation period between populations, two-way ANOVA with time point and population as independent variables was followed by Tukey's post hoc test for multiple pairwise comparisons. Transcript levels of *Ae. aegypti* immune pathway genes were compared to the control group using the Wilcoxon test. All statistical analyses were performed using JMP Pro (www.jmp.com) and GraphPad Prism 9 (www.graphpad.com). The figures were made with GraphPad Prism 9.

## Results

### Estimation of vector competence in different *Ae. aegypti* populations under DENV-2 infection

Vector competence studies were conducted at 10 days after infection (dpi) with three *Ae. aegypti* populations from the Americas, Vilas do Atlântico (VDA), California (CA), and Vero. All populations were
fed with blood containing $7.23 \pm 0.62 \log \text{PFUe/ml}$ of DENV-2. On average, freshly fed VDA, CA and Vero mosquitoes imbibed $4.29 \pm 0.43$, $4.23 \pm 0.5$, and $4.03 \pm 0.53 \log \text{PFUe/ml}$ of DENV-2, respectively. The virus titers in bloodmeals did not significantly differ between populations ($p > 0.05$). The feeding rates of controls for VDA, CA, and Vero were $74.7\%$ (112/150), $66.7\%$ (100/150), and $81\%$ (81/100), respectively, while the feeding rates with virus-containing blood for VDA, CA, and Vero were $38.4\%$ (93/250), $44\%$ (107/250), and $54.4\%$ (133/250). The CA population had lower clean blood feeding rate than Vero ($p = 0.014$). The Vero population had higher feeding rates with virus-containing blood compared to VDA ($p < 0.001$) and CA ($p = 0.03$) populations.

The infection rate varied among the three populations tested from a low of 50$\%$ in VDA ($n = 18$) followed by 72.4$\%$ in CA ($n = 29$) and 82.8$\%$ in Vero ($n = 29$) (Table 1). The two populations from North America, CA, and Vero, had higher DENV infection rates than VDA, but differences were only significant between VDA and Vero ($p = 0.024$).

<table>
<thead>
<tr>
<th>Population</th>
<th>Number tested</th>
<th>Infection rate</th>
<th>Dissemination rate</th>
<th>Transmission rate</th>
<th>Transmission efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vilas do Atlântico (VDA)</td>
<td>18</td>
<td>9 (50.0%)</td>
<td>4 (44.4%)</td>
<td>1 (25.0%)</td>
<td>5.6$^{g}$</td>
</tr>
<tr>
<td>California (CA)</td>
<td>29</td>
<td>21 (72.4%)</td>
<td>17 (81.0%)</td>
<td>5 (29.4%)</td>
<td>17.2$^{g}$</td>
</tr>
<tr>
<td>Vero</td>
<td>29</td>
<td>24 (82.8%)</td>
<td>21 (87.5%)</td>
<td>15 (71.4%)</td>
<td>51.7$^{h}$</td>
</tr>
</tbody>
</table>

Levels not connected by the same letter are significantly different.

The dissemination rate in the VDA population was 44.4$\%$ ($n = 9$), while the CA and Vero populations had dissemination rates of 81.01$\%$ ($n = 21$) and 87.5$\%$ ($n = 24$), respectively (Table 1). Although the CA and Vero populations had higher dissemination rates than VDA, similar to infection rates, the only significant difference was between the Vero and VDA populations ($p = 0.02$).

For the transmission rate, the data indicated rates of 25$\%$ ($n = 4$), 29.4$\%$ ($n = 17$) and 71.4$\%$ ($n = 21$) in the VDA, CA and Vero populations, respectively (Table 1). The Vero population had the highest transmission rate, which was significantly greater than the transmission rate of the CA population ($p = 0.028$).

The transmission efficiency (TE) (number with viral RNA detected in the saliva/ number of blood-engorged mosquitoes) was 5.6$\%$, 17.2$\%$ and 51.7$\%$ in the VDA, CA and Vero populations, respectively (Table 1). The Vero population had the highest TE and was significantly different to VDA ($p = 0.001$) and CA ($p = 0.01$) populations.
Titors of DENV-2 at 10 dpi in mosquito body samples were 5.32 ± 0.45, 5.41 ± 0.85, and 5.83 ± 1.04 log PFUe/ml in the VDA, CA, and Vero populations, respectively (Fig. 1A). The VDA population had the lowest DENV-2 titer, which was significantly different when compared to average titors from Vero (p = 0.009) but not CA (p = 0.13).

Detection of DENV-2 titer in the early extrinsic incubation period (EIP)

The eclipse phase occurred in all three populations; DENV-2 titer decreased at 1 dpi compared to the infectious bloodmeal (Fig. 1B). The VDA, CA, and Vero populations had titers of 3.28 ± 0.19, 2.69 ± 0.15, and 3.01 ± 0.07 log PFUe/ml, respectively. DENV-2 titer continued to decrease at 2 dpi in the VDA (1.31 ± 0.71 log PFUe/ml) and Vero (1.99 ± 0.31 log PFUe/ml) populations but not in CA (2.77 ± 0.33 log PFUe/ml) population. The CA population had a relatively higher titer at 2 dpi and was significantly different from the VDA population (p = 0.005). At 3 dpi, DENV titers increased in all populations, as the VDA, CA, and Vero populations had titers of 2.62 ± 0.33, 4.88 ± 0.16, and 3.24 ± 0.79 log PFUe/ml). The CA population had the highest DENV-2 titer and was significantly different from the Vero population (p = 0.001) and the VDA population (p < 0.0001), which had the lowest DENV-2 titer.

Alterations in transcript level of Ae. aegypti immune pathway genes early in the EIP after DENV-2 infection

The transcripts of several Ae. aegypti genes from multiple immune pathways were quantified during the first three days after DENV-2 infection. Compared to blood-only control groups, mosquitoes fed with DENV-2 demonstrated variation in immune transcript levels among populations and time points (Table 2).
Table 2
Relative immune-related transcript fold change during the first three days post-feeding between the virus-infected group and blood-only group.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Vilas do Atlântico (VDA)</th>
<th>California (CA)</th>
<th>Vero</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
<td>Day 3</td>
</tr>
<tr>
<td>Rel-1A</td>
<td>0.79 ± 0.08</td>
<td>0.59 ± 0.23</td>
<td>0.86 ± 0.06</td>
</tr>
<tr>
<td>Rel-1B</td>
<td>0.94 ± 0.14</td>
<td>1.22 ± 0.27</td>
<td>1.05 ± 0.23</td>
</tr>
<tr>
<td>Rel-2</td>
<td>1.04 ± 0.24</td>
<td>1.78 ± 0.75</td>
<td>1.53 ± 0.22</td>
</tr>
<tr>
<td>Dome</td>
<td>1.50 ± 0.87</td>
<td>0.87 ± 0.16</td>
<td>0.53 ± 0.21</td>
</tr>
<tr>
<td>ATG5</td>
<td>0.90 ± 0.12</td>
<td>1.27 ± 0.80</td>
<td>0.83 ± 0.54</td>
</tr>
<tr>
<td>Dronc</td>
<td>0.94 ± 0.14</td>
<td>1.16 ± 0.54</td>
<td>0.67 ± 0.27</td>
</tr>
<tr>
<td>IAP-1</td>
<td>1.25 ± 0.21</td>
<td>1.93 ± 1.42</td>
<td>1.52 ± 0.73</td>
</tr>
<tr>
<td>Ago-2</td>
<td>0.75 ± 0.04</td>
<td>0.85 ± 0.30</td>
<td>2.35 ± 0.92</td>
</tr>
</tbody>
</table>

The asterisk (*) indicates statistical difference between blood-fed and virus infected mosquitoes.

In the Toll pathway, Rel-1A, which functions as a transcription factor, did not show significant differences between DENV-2 infected mosquitoes and blood-fed mosquitoes from the VDA and Vero populations. However, in the CA population, Rel-1A transcript had higher levels at 1 dpi (2.68 ± 0.68, p = 0.02) and 3 dpi (3.32 ± 0.56, p = 0.049) but decreased at 2 dpi (0.45 ± 0.21, p = 0.043). Rel-1B, another Toll pathway downstream transcription factor, showed a similar pattern, as the CA population had significantly higher transcript levels at 1 dpi (2.30 ± 0.58, p = 0.021) and 3 dpi (5.13 ± 1.1, p = 0.034) compared to the other populations.

Expression of Rel-2, a gene involved in the IMD pathway, was downregulated at 2 dpi in the Vero population (0.49 ± 0.07, p = 0.021) but not in the other two populations. The JAK/STAT pathway receptor Dome did not vary in transcript level after infection in any population. Autophagy related 5 (ATG5) which is involved in extension of the phagophoric membrane in autophagic vesicles in the autophagy pathway, only showed a higher transcript level at 3 dpi in the CA population (1.71 ± 0.45, p = 0.043) and Vero population (1.33 ± 0.10, p = 0.021). The apoptosis genes Dronc (1.68 ± 0.34, p = 0.043) and IAP-1 (1.93 ± 0.95, p = 0.043), and RNAi pathway gene Ago-2 (4.44 ± 2.67, p = 0.042) also showed significant increases
in transcripts in the CA population at 3 dpi. In contrast, the transcription of IAP-1 (0.83 ± 0.04, p = 0.004) was downregulated in the Vero population at 3 dpi.

**Toll pathway gene transcript levels between Ae. aegypti populations after blood-feeding**

To examine population-level variation in the immune system, we compared transcripts of selected immunity genes between the three populations after blood feeding but without virus. Because the Vero population was the most susceptible to infection, this population was used to normalize relative gene expression and to further calculate the expression differences. Evaluation of the Toll pathway transcription factor Rel-1A revealed the VDA population had the relatively highest level (2.33 ± 1.39) and was significantly different from CA (0.87 ± 0.29, p = 0.007) and Vero (1.03 ± 0.27, p = 0.017) at 2 days post blood feeding (dpb) (Fig. 2A). For another Toll pathway transcription factor, Rel-1B, the VDA population had significantly higher transcript levels at 2 dpb (2.39 ± 0.85) compared to the CA (0.38 ± 0.14, p = 0.01). Moreover, at 3 dpb, the transcript level of Rel-1B remained the highest in the VDA population (3.53 ± 2.29) and was significantly greater than the transcript level for the CA (1.11 ± 0.63, p = 0.005) and Vero (1.16 ± 0.67, p = 0.003) populations (Fig. 2B).

Besides the transcripts from Toll pathway, Rel-2 from IMD pathway and Dome from JAK/STAT pathway were tested (Table S2). The Rel-2 transcript in CA population was downregulated at 2 dpb (0.39 ± 0.23) and upregulated at 3 dpb (3.92 ± 2.9) compared to Vero population (2 dpb: 1.01 ± 0.2, p = 0.02; 3 dpb: 1.03 ± 0.31, p = 0.018). The Dome transcript in CA population (5.45 ± 0.5) was significantly higher than Vero at 3 dpb (1.09 ± 0.57, p = 0.04).

**Microbiota differences between Ae. aegypti populations**

Analysis of the 16S rRNA gene amplicons revealed that colonies cultured from mosquito samples were dominated by *Asaia bogorensis* and *Asaia platycodi*, (Alphaproteobacteria) and *Elizabethkingia anophelis* (Flavobacteria) in the VDA population. *Asaia platycodi* and *Elizabethkingia anophelis* sequences were amplified from the Vero population as well, while the CA population produced sequences belonging only to the genus *Elizabethkingia* (Fig. 3A, Table S3, S4).

From fungal 18S rRNA gene libraries, a total of eight species were identified among all three populations. The VDA population generated the most diversity (Fig. 3B, Table S5, S6) with all eight fungal species identified, three of which were classified as the yeasts (*Sacharomycetes* Starmerella etchellsii, *Candida zeylanoides*, and *Meyerozyma guilliermondii*). Four species were discriminated as filamentous fungi (*Basidiomycetes*): *Pseudotremella allantoinivorans*, *Rhodosporidiobolus colostrii*, *Tilletiaria anomala* and *Wallemia canadensis*. One species was categorized as mold-like fungi (*Mucoromycota*): *Paraglomus occulatum*. Four sequences were generated from the Vero population, including two yeast species (*C. zeylanoides*, and *M. guilliermondii*) and two filamentous fungi (*T. anomala*, and *W. canadensis*). *Candida zeylanoides*, *Tilletiaria anomala* and *W. canadensis* were the only species represented in fungal sequences from the CA population.

Flavivirus gene libraries were generated only from the VDA and Vero populations (Fig. 3C, Table S7) with their sequences matching to previously described flavivirus isolates from *Ae. aegypti* in Argentina.
(Flaviviridae sp. isolates Arg/Fla/Aeae) and from a sand fly Phlebotomus (Phlebotomus-associated flavivirus).

**Discussion**

Vector competence is influenced by multiple factors, including the interaction between mosquito genotypes and microbiota [9]. The *Ae. aegypti* populations from different geographic locations are genetically dissimilar [47] and exhibit variability in DENV vector competence [9, 48]. To identify the factors associated with vector competence, it is critical to understand the transmission cycle and develop potential control methods. Here we provided a study of screening the possible interaction between virus, mosquito genotypes and microbiota. The results of the vector competence for DENV in three geographically distinct *Ae. aegypti* populations, along with variability in the immune response and in microbiota and support for multiple mechanisms underlying variation in mosquito susceptibility to the virus are discussed.

To evaluate vector competence, characterizing rates of infection, dissemination, and transmission is crucial. In our study, despite small sample sizes, the VDA population had significantly lower infection, dissemination rates and viral titer at 10 dpi compared to Vero population. On the other hand, despite VDA population having no statistical difference to the CA population at 10 dpi, the viral titer was significantly less at 2 and 3 dpi between VDA and CA populations. Considering all the data we collected, the VDA population revealed a relatively high refractoriness to DENV-2 compared to the other two populations (Table 1, Fig. 1). In contrast, although the CA population showed high rates of infection and dissemination, its transmission rate was relatively low. The Vero population had similar susceptibility to DENV-2 as CA but a significantly higher transmission rate, suggesting that this population is the better vector. Since the three geographically distinct populations represented different susceptibilities to DENV, we defined them as populations with unique backgrounds and thus they will be used to further investigate the potential factors involved [49]. Interestingly, the feeding rate of *Ae. aegypti* from this study was low, especially the VDA population (Table 1), possibly due to fewer years in colony from the field and slower adaptation to laboratory conditions [50].

The eclipse period early in the EIP indicates the virus titer drops in the mosquito during ingestion [48] which might be indicative of antiviral response in the mosquito, although this is speculative as the mechanism is not well understood. All three populations in this study exhibited an eclipse phase (Fig. 1B). Interestingly, the viral titer in the CA population remained similar between 1 dpi and 2 dpi and then increased significantly at 3 dpi, reaching titers in CA more than 40-fold and 180-fold greater compared to Vero and VDA, respectively. The high viral titer in the CA population likely explains upregulation of several immune-related transcripts at 3 dpi (Table 2). The NF-κB-like transcription factor Rel-1A and its co-activator Rel-1B are downstream factors in the Toll pathway, which receive activation signals to translocate into the nucleus to initiate transcription during infection with pathogens [51], and both signal transduction genes were upregulated in the CA population at 3 dpi. Rel-1A gene transcription increased under DENV infection in *Ae. aegypti* at 10 dpi [16] but not Rel-1B. However, the temporal and
population differences might cause the immune response to vary. Because the Toll pathway is associated with anti-viral response in *Ae. aegypti* [16, 52, 53], increasing Rel-1 transcripts in the CA population might be a response to the higher viral titer. The Ago-2 transcript was also upregulated at 3 dpi in the CA population. The Ago-2 protein is the effector in the RNA-induced silencing complex (RISC) and uses a guide strand to target viral RNA for cleavage and degradation via the RNAi pathway [54, 55]. In *Ae. aegypti*, silencing Ago-2 gene expression decreased the incubation time of DENV and increased the transmission rate [11]. The RNAi biological response also limited the replication of CHIKV in *Ae. aegypti* [12] and Onyong’nyong virus in *Anopheles gambiae* [10], supporting the assertion that the RNAi pathway is one of the most important anti-viral responses in mosquitoes. Considering the CA population did not show the highest titer at 10 dpi, it might suggest that both Toll and RNAi pathway played the antiviral roles once induced from the high viral load at 3 dpi.

Gene transcripts of the apoptosis and autophagy pathways were altered at 3 dpi in both the CA and Vero populations. Apoptosis has been identified as an antiviral response in insects [56]. Moreover, previous studies showed apoptosis pathway genes were highly expressed in a DENV-2 refractory strain of *Ae. aegypti* [57] and silencing the pro-apoptotic gene Dronc allowed the DENV-2 infection rate to increase [21]. Apoptosis activation limits ZIKV and DENV-2 proliferation in the midguts of *Ae. aegypti* [58]. Given the significant increase in Dronc expression observed in the CA population at 3 dpi, these findings might suggest that the high DENV titer at the same time point for this population triggered an increase in the apoptosis pathway. However, our study also showed upregulation of expression of IAP-1 in the CA population suggesting possible negative feedback to the highly activated apoptosis pathway. The autophagy pathway has also been shown to serve an immunity function in insects [59]. The ATG5 gene product is an initiator of autophagosome formation [60, 61] and a key component in the autophagy pathway. Several ATG genes were upregulated under DENV infection in an *Ae. aegypti* refractory strain [22, 62], and autophagy activation decreased DENV titer in a recent study [23]. However, the VDA population, which our data implicate as being refractory to DENV-2, did not exhibit significant changes in ATG5 expression at any time point, though the highest expression at 2 dpi corresponded to a low DENV titer. Conversely, ATG5 expression in CA and Vero populations was significantly upregulated at 3 dpi corresponding to time points where mosquitoes from these populations had high DENV titers. Our results, in combination with the literature, suggest that interactions between apoptosis, autophagy, and DENV are complex and warrant further investigation. Moreover, the apoptosis and autophagy pathway transcripts were altered in both CA and Vero populations suggesting they might not be the major antiviral response, at least in the three populations we tested.

The IMD and JAK/STAT pathways have been previously characterized as antiviral pathways in insects [17–20]. In this study, however, transcripts from neither pathway were upregulated in any of the three populations at any time point, but Rel-2 transcription was downregulated at 2 dpi in the Vero population. Although the CA population had increased transcription of genes in several pathways, the uniqueness of the involvement of the Toll and RNAi pathways in infection in the CA population was noteworthy. Considering that this population had high infection and dissemination rates but a relatively low
transmission rate, both the Toll and RNAi pathways might play a role in SGIB and SGEB. Future studies are necessary to specifically test this hypothesis.

Surprisingly, the refractory population VDA did not have any significant differences in transcription of immune-related genes in the first three days of DENV-2 infection. We, therefore, compared the basal transcript levels of genes in the Toll pathway between populations after blood feeding (Fig. 2). The Rel-1A and Rel-1B transcripts in the VDA population were highly expressed compared to the other two populations at 2 and 3 dpb, which suggested the possibility that blood triggered the Toll pathway in the VDA population without virus treatment. Besides its antiviral function, Rel-1 was implicated as an important factor in regulating the antifungal and antibacterial immune responses via the Toll pathway in *Ae. aegypti* [63–65]. Additionally, a symbiotic bacterium induced reactive oxygen species through the Toll pathway in *Ae. aegypti* and limited DENV proliferation [66], indicating the possibility that microbiota influence vector competence indirectly by activating the Toll pathway [26].

Since the microbiota composition is known to change after blood feeding [67, 68] and because the Rel-1A and Rel-1B transcripts were upregulated at 2 and 3 dpb in VDA population, we focused on post-blood feeding differences in microbiota between populations. The microbiota screening revealed the variation in the VDA population but limited species abundance in the CA population (Fig. 3, Table S3-7).

In bacteria screening, *Asaia* (*Rhodospirillales*) sequences were identified in VDA and Vero populations but not in CA. *Asaia* are commonly present in different mosquito species [69, 70]. Bacteria in this genus are known to interact with resident microbiota [71, 72] and to influence vector metabolism [73]. Particularly, *Asaia* was found to modulate midgut pH through glucose metabolism in the *Anopheles* vector, which in turn promotes Plasmodium microgametogenesis (i.e., development of male gametes), a critical step in the malaria-parasite life cycle that when increased enhances infection of the mosquito midgut [74]. In contrast, *Asaia* was also reported to negatively impact Plasmodium by activating immune genes in two *Anopheles* species that triggered anti-plasmodium responses [75]. However, whether *Asaia* is involved in the response to DENV infection in *Ae. aegypti* is still unclear.

*Elizabethkingia anophelis* (*Flavobacteriales*), a bacterium originally isolated from *Anopheles gambiae* [76] and commonly found in *Aedes aegypti* mosquitoes [77, 78] was recognized in all three population through Blast analyses. This bacterium was linked to the mitigation of iron stress in *Anophles gambiae* during blood meal intake by digesting red blood cells [79]. *E. anophelis* has also been linked to Zika virus decline in infected *Aedes albopictus* mosquitoes, though the mechanism(s) underlying this negative interaction was not determined [80]. Even though *E. anophelis* was detected in all three populations, it is unknown whether this bacterium was involved in the interplay with dengue virus and *Ae. aegypti* from this study.

Although to date, not many of the fungal species have been identified in *Ae. aegypti* [46, 70], some of the fungal genera we recognized have been discovered broadly among different insects, including those in the order *Diptera*, such as *Drosophilids*, as well as social insects in the order *Hymenoptera* [81]. In the VDA population, two unique yeasts from the *Saccharomycetales* order, *S. etchellsii* and *R. colostrii* were
detected, along with the mycorrhizal fungus *P. occulatum* (*Paraglomerales*) [82] and *P. allantivorans* (*Tremellales*), described as a parasite of other fungi [83]. The *M. guilliermondii* (*Saccharomycetales*), *T. anomala* (*Georgescheriales*) and *W. canadensis* (*Wallemiales*) were shared among the VDA and Vero populations. The data indicate that two fungal species, *C. zeylanoides* (*Saccharomycetales*) and *W. canadensis*, appeared in all three populations. The discovered species are classified in orders and subphyla of fungi that have already been identified in mosquitoes and their larval habitats through next generation sequencing [84, 85]. Considering the geographical distance of the three populations that were investigated, it is possible that the species in common were acquired from the climate-controlled room in which the mosquitoes were reared [24].

Two insect-specific flaviviruses, Phlebotomus-associated flavivirus and Flaviviridae sp. Arg/Fla/Aeae/02 isolate, were identified in the VDA population, while only Phlebotomus-associated flavivirus was detected in the Vero population. However, none of the flaviviruses were found in the CA population. Both strains were reported previously in different *Ae. aegypti* populations [86, 87]. Although not the same species we identified, insect-specific flaviviruses have been shown to interact with the mosquito immune system and result in a significant reduction of arbovirus-co-infection in vitro [88]. While microbiota have been shown to potentially effect vector competence [29, 35], evidence to connect virus infection in the mosquito with the species identified from the three populations is lacking.

**Conclusions**

Here we provided support for the potential involvement of immune pathways and microbiota in shaping variation in vector competence for DENV in *Ae. aegypti* but added to existing knowledge by showing that pathway activation and components of the microbiota differ by geographic origin of the mosquito population. Between the three geographically distinct *Ae. aegypti* populations, the CA population had a low transmission rate and might be associated with the upregulation of immune-related transcripts, especially the Toll and RNAi pathways. The competent Vero population had altered transcription of genes in the apoptosis and autophagy pathways, suggesting minor effects on antiviral functions as the titer was the highest among all three populations at 10 dpi. Moreover, the infection, dissemination and transmission rates were also higher than the other two populations. Although the refractory VDA population did not show any transcriptional differences after infection, the Rel-1 basal level was higher than that of the other populations after blood ingestion, suggesting this population might have more efficient antiviral signaling. Additionally, VDA contained the most diverse microbiota species, which may contribute to its reduced vector competence. Deciphering the components of mosquito microbiota and targeting the microbial candidates with particular attributes, could contribute to reducing the mosquito borne diseases through novel symbiotic control approaches [89–91]. All in all, we offered a comprehensive study to identify the possible factors that influence resistance to DENV-2 in *Ae. aegypti* and provide potentially new prospective control methods to develop.

**Declarations**
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Authors’ contributions

T-YC, JB, DM, and CTS conceived and designed the experiments. T-YC, JB, and CTS performed the experiments. T-YC, JB, DM, and CTS analysed the data. T-YC, JB, DM, and CTS wrote the manuscript. All authors contributed to the manuscript and approved the submitted version.

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Availability of data and materials

No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

References


Figures

Figure 1

Dengue virus titer variation among *Ae. aegypti* populations. (A) The DENV-2 Pfue/ml (Log) at 10 days post-infection. (B) The DENV-2 Pfue/ml (Log) titers in the first three days in three populations. The asterisk (*) indicates statistical difference, * p-value < 0.05.** p-value < 0.01.
Figure 2

The Rel-1A and Rel-1B relative fold change in transcription between blood-fed *Ae. aegypti* populations. (A) The Rel-1A expression fold change compared to the Vero population after blood feeding. (B) The Rel-1B expression fold change compared to the Vero population after blood feeding. The asterisk (*) indicates statistical difference, * p-value < 0.05.
Figure 3

Venn diagram for microbiota screening in three populations post blood feeding. (A) Bacteria 16S rRNA library. (B) Yeast/fungi 18S rRNA library. (C) Flaviviruses NS5 library.

Supplementary Files

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