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Vector competence and innate immune responses to dengue virus infection in selected laboratory and field-collected *Stegomyia aegypti* (=*Aedes aegypti*)

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Abstract

Control of dengue virus (DenV) transmission, primarily based on strategies to reduce populations of the principle vector *Stegomya aegypti* (= *Aedes aegypti*) (Diptera: Culicidae), is difficult to sustain over time. Other potential strategies aim to manipulate characteristics such as vector competence (VC), the innate capacity of the vector to transmit the virus. Previous studies have identified genetic factors, including differential expression of apoptosis-related genes, associated with the refractory and susceptible phenotypes in selected strains of *S. aegypti* from Cali, Colombia. The present study was designed to evaluate the variability of VC in selected strains against different DenV serotypes and to determine whether field-collected mosquitoes respond similarly to selected laboratory strains in terms of enhanced or reduced expression of apoptosis-related genes. Vector competence differed between strains, but did not differ in response to different DenV serotypes. Differences in VC were observed among mosquitoes collected from different localities in Cali. The overexpression of the pro-apoptosis genes, *caspase 16* and *Aedronc*, was conserved in field-collected refractory mosquitoes and the selected laboratory refractory strain. The results suggest that the apoptosis response is conserved among all refractory mosquitoes to inhibit the development of all DenV serotypes.

Keywords

Apoptosis; caspases; dengue virus; innate immunity; vector competence

Introduction

Dengue virus (DenV) is a single positive stranded RNA virus transmitted by mosquitoes, especially *Stegomyia aegypti* (= *Aedes aegypti*) and *Stegomyia albopicta* (= *Aedes albopictus*), that manifests as dengue fever, a human disease for which there is no specific treatment. Although there have been major advances in vaccine development, no vaccines are 100% protective. Dengue is endemic in more than 100 countries in tropical and

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subtropical areas where 2.5 billion people are at risk and accounts for a global burden of 390 million infections per year, of which 96 million are symptomatic (Bhatt *et al.*, 2013). In the Americas, dengue incidences have increased 30-fold over the last 50 years (World Health Organization, 2012).

Dengue control has traditionally focused on the reduction of vector densities through the application of insecticide and the elimination of mosquito larval habitats. However, the effectiveness of chemical control has been limited by the development of insecticide resistance, and the high costs of establishing and maintaining vector control programmes (Guzman & Harris, 2015). New strategies are therefore needed to decrease transmission. Advances in molecular biology and the availability of genome databases have opened new perspectives in the control of vectors, such as the genetic manipulation of mosquitoes (Fu *et al.*, 2010), the development of transmission-blocking vaccines (Coutinho-Abreu & Ramalho-Ortigao, 2010) and the design of new insecticides. Some of these efforts are focused on modifying insect characteristics that determine their efficiency as vectors, such as vector competence (VC), the intrinsic ability of a vector to transmit a pathogen (Fu *et al.*, 2010). However, the manipulation of an insect's VC requires extensive knowledge of vector–pathogen interactions.

Vector competence depends on intrinsic genetic characteristics that influence the compatibility of the virus and vector (Fu *et al.*, 2010; Schneider *et al.*, 2011; Wise de Valdez *et al.*, 2011). Incoming pathogens must avoid being eliminated by the innate immune response of the vector. Pattern recognition receptors (PRRs) in the insect recognize conserved pathogen-associated molecular patterns (PAMPs) on the surface of pathogens and activate multiple immune-related pathways that ultimately result in phagocytosis, melanization and the expression of reactive oxygen intermediates and antimicrobial peptides (AMPs) (Paradkar *et al.*, 2012). The major pathways include the Toll (Fu *et al.*, 2010), immune deficiency (Xi *et al.*, 2008; Behura *et al.*, 2011), RNA interference (RNAi) (Sánchez-Vargas *et al.*, 2009), Janus kinase–signal transducer and activator of transcription (JAK-STAT) (Kleino *et al.*, 2005; Souza-Neto *et al.*, 2009) pathways, and autophagy and apoptosis (Behura *et al.*, 2011). Although it has been traditionally considered that specific pathways respond to specific pathogens, there are shared molecules within and between multiple pathways that result in a generalized activation of multiple pathways by a single pathogen.

Dengue virus is an intracellular pathogen and, as such, is not exposed directly to many of the effector molecules of the innate immune system. Nonetheless, DenV infection in mosquitoes is recognized by the vector, resulting in the activation of the Toll, immune deficiency (Xi *et al.*, 2008), JAK-STAT (Souza-Neto *et al.*, 2009) and RNAi (Sánchez-Vargas *et al.*, 2009) pathways, autophagy and apoptosis (Ocampo *et al.*, 2013) that control, regulate or modulate DenV establishment and replication. There is significant molecular interplay between vector and virus that may increase or decrease immune responses and the subsequent success or failure of DenV to replicate within, and be transmitted by, an individual mosquito.

High variability in the VC of mosquitoes collected in Cali, Colombia has been previously demonstrated (Ocampo & Wesson, 2004), and strains with different levels of VC for DenV-2

have been selected (Caicedo *et al.*, 2013) in order to understand potential molecular mechanisms associated with susceptible (Cali-S) or refractory (Cali-MIB) *S. aegypti.* Differences between Cali-S and Cali-MIB in the expression of specific genes, especially innate immune-related genes associated with apoptosis (Baron *et al.*, 2010; Ocampo *et al.*, 2013) have been demonstrated and the contributions of these genes to the phenotypes have been confirmed using RNAi silencing assays (Ocampo *et al.*, 2013). These studies evaluated the innate immune response to DenV-2 and questioned whether this was serotype-specific or a general response to all DenV serotypes. The study described herein was performed to determine whether the observed responses associated with the VC of Cali-MIB were a product of the laboratory selection process itself or were mechanisms conserved and maintained in wild mosquitoes.

Materials and methods

Mosquito strains

The selected *S. aegypti* strains, Cali-S and Cali-MIB, were established in the laboratory facilities at the Centro Internacional de Entrenamiento e Investigaciones Médicas (CIDEIM) from larvae collected from several sites around the city of Cali, Colombia. The phenotypes were selected after exposure to DenV-2 New Guinea C (NGC) through isofamily selection as previously described (Caicedo *et al.*, 2013). The selected strains were maintained in the laboratory and given exposure to DenV every two generations to maintain selection pressure.

Stegomyia aegypti larvae were collected from six localities around Cali, including Mariano Ramos, Valle Grande, Navarro la Y, Siloe, Antonio Nariño and Paso del Comercio, all of which are at least 5 km from one another. Larvae were collected from small containers in public places; all larvae collected within the same neighbourhood were combined and named by locality. Collected larvae were maintained at the CIDEIM insectary under standard rearing conditions at 27 ± 2 °C, 56% relative humidity and an LD photoperiod of 12 : 12 h. The larvae were kept in plastic containers at a density of ~ 300 larvae in 2 L of dechlorinated water and were fed with Tetramin® (Spectrum Brands, Inc., Middleton, WI, U.S.A.). Adults were provided with 10% sucrose solution *ad libitum*. Mosquitoes from each locality were allowed to mate and oviposit, and the subsequent experiments were carried out with mosquitoes from the F_2 and F_3 generations from each locality.

Viral propagation, experimental infections and VC determination

Cali-S and Cali-MIB females were exposed to four different DenV reference serotypes [DenV-1 Hawaii; DenV-2 New Guinea C (NGC), DenV-3H-87 (Philippines 56), DenV-4H-241 (Philippines 56)], all of which were supplied by Colorado State University in 2000. Levels of VC in the mosquitoes collected from different locations in Cali were determined using the reference strain DenV-2 NGC to compare the results of the present study with those reported previously in the selected strains (Ocampo *et al.*, 2013).

Each DenV serotype was grown in *S. albopicta* C6/36 HT cells. Infected cells were incubated for 14 days at 32 °C in L-15 medium supplemented with 2% fetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine (Higgs *et al.*, 1996). The virus and cells

were harvested and collected in 15-mL conical centrifuge tubes. The virus suspension was mixed (1 : 1) with defibrinated rabbit blood, and adult female mosquitoes were exposed to this mixture through an artificial feeder using a pig intestine membrane. In order to determine viral concentration [median tissue culture infective dose per mL ($TCID_{50}$/mL)], aliquots of the infected cell/blood suspension pre- and post-infection, as well as rabbit blood, were titrated to assess the amount of virus to which female mosquitoes were exposed (Bennett *et al.*, 2005).

For each infection, approximately 50 females (aged 5–7 days) from each strain (Cali-MIB and Cali-S) were starved for 12 h and then exposed to the infectious bloodmeal. All feedings were performed in a Biosafety Level 2+ (BSL-2+) laboratory at CIDEIM. Mosquitoes were allowed to feed on the viral blood mixture for 2 h. Each individual serotype experiment was conducted four times. Fully engorged females were transferred to a separate cage and maintained for 15 days, the length of the extrinsic incubation period of the virus in the vector, under standard conditions as described above. Subsequently, the presence of disseminated virus in the head and abdomen of each mosquito was evaluated using indirect immunofluorescence (IIF) as described by Schoepp & Beaty (1984). Antibodies used in this study (anti-DenV-1 D2-1F1-3, anti-DenV-2 3H5-1-21, anti-DenV-3 D6-8A1-12, anti-DenV-4 1H10-6-7) were supplied by the Centers for Disease Control and Prevention (CDC), Puerto Rico.

Mosquitoes with positive head smears as determined by IIF were categorized as the susceptible phenotype. Mosquitoes with negative head smears were further dissected and their midguts assessed for the presence of DenV using IIF. Mosquitoes that were negative for DenV in both head and midgut smears were classified as refractory via a midgut infection barrier (MIB) phenotype, and those with negative head but positive midgut smears were classified as refractory via a midgut escape barrier (MEB) phenotype.

Vector competences were measured in the selected strains, Cali-S and Cali-MIB, and in the field-collected mosquitoes from six different localities in Cali.

Differences between the Cali-S and Cali-MIB strains in VC for each DenV serotype were measured using a binomial regression model to estimate the numbers of mosquitoes with susceptible and refractory phenotypes. The number of susceptible mosquitoes in each strain was independent of the number of refractory mosquitoes. The model used was: $\text{logit}(p) = \beta_0 + \beta_{1i} x_{1i} + \beta_{2j} x_{2j}$, where β_0 represents the intercept, β_{1i} represents the log-odds ratios for the effects of strains ($i = 1, 2$; 1 = Cali-MIB; 2 = Cali-S), β_{2j} represents the log-odds ratios for the effects of each serotype ($j = 1, 2, 3, 4$; 1 = DenV-2 (reference); 2 = DenV-1; 3 = DenV-3; 4 = DenV-4); x_{1i} represents the strain, and x_{2j} represents the serotype.

To determine whether there were differences in VC for DenV-2 among the field-collected *S. aegypti* from different neighbourhoods, chi-squared tests were performed to compare observed and expected frequencies of mosquitoes in relation to susceptible and refractory MIB phenotypes.

Gene expression studies

As previous studies had identified differential expression of apoptosis-related genes in Cali-S and Cali-MIB females (Ocampo *et al.*, 2013), the expression levels of selected genes in pools of midguts of F_2 or F_3 generations from a sample of mosquitoes collected at each of the localities described above were measured.

Mosquitoes were exposed to either blood or blood + DenV-2, as previously described (Ocampo *et al.*, 2013). Midguts of engorged females were dissected in diethylpyrocarbonate (DEPC)-treated water (Life Technologies, Inc., Carlsbad, CA, U.S.A.) at 0 h, 24 h, 36 h and 48 h post-feeding, and stored in pools of 10 midguts in 50 μL of RNAlater (Life Technologies, Inc.). Samples were stored for 24 h at 15 °C, 24 h at 0 °C and then at −70 °C to prevent the formation of crystals. Midguts were gradually thawed, RNAlater was removed from each vial and midgut RNA was extracted using the RNeasy® Mini Kit (Qiagen, Inc., Valencia, CA, U.S.A.). Total RNA was quantified using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies LLC, Wilmington, DE, U.S.A.).

For cDNA synthesis, 100 ng of total RNA per time-point was reverse-transcribed in 20-μL reaction mixtures containing 5× first-strand buffer [50 mM Tris-HCl (pH 8.3)], 0.1 M DTT, 10 mM of each dNTP, 50 ng of Oligo(dT) primer (5′-CGGGCAGTGAGCGCAACGTTTTTTTTTTTTTT-3′) and 200 units of Superscript II Reverse Transcriptase (Life Technologies Corp., Grand Island, NY, U.S.A.). Reverse transcription was conducted at 42 °C for 50 min and then at 70 °C for 15 min. The resulting cDNAs were used in subsequent real-time quantitative polymerase chain reactions (qPCRs) using the primers in Table 1.

Gene expression was measured in 12.5-μL reactions prepared with 1 μL of cDNA product, 1 μL of each primer (10 μM) and 6.25 μL of SYBR Green ERTM Express (Life Technologies Corp.) in a Bio-Rad CFX96™ Real-Time System (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.). The conditions were: 95 °C for 2 min, followed by 35 cycles of 95 °C for 10 s, 57 °C or 60 °C for 15 s (Table 1) and 72 °C for 20 s. Two biological replicates (three in Antonio Nariño samples) were analysed for collections from each locality and all qPCR reactions were performed in triplicate. The expression of each gene of interest was analysed in each of the F_2/F_3 generations collected from each of the different localities. Expression levels were normalized using an internal control [β-actin (AAEL001928)] to generate ΔCt values.

Gene expression was compared between strains (refractory vs. susceptible strain) after exposure to blood or blood + DenV-2 using $2^{-\Delta Ct\ \text{refractory strain}}/2^{-\Delta Ct\ \text{susceptible}}$ strain (Schmittgen & Livak, 2008). Results are presented as the mean ± standard deviation.

A multiple linear regression to identify differences in gene expression in the mosquitoes collected from the different localities was performed on the qPCR data, with the response variable being ΔCt = Ct (sample) − Ct [calibrator (β-actin)] and the independent factors being time, treatment (blood vs. blood + DenV-2) and strain. Robust standard errors were used to account for correlations between replicates. The model was: $y = \beta_0 + \beta_{1t} x_{1t} + \beta_{2i} x_{2i} + \beta_{3j} x_{3j} + \varepsilon_{tij}$, where y represents ΔCt actin, β_0 represents the intercept, β_{1t} represents the

coefficient for the effects between times (t = 1, 2, 3, 4; 1 = 0 h; 2 = 24 h; 3 = 36 h; 4 = 48 h), β_{2i} represents the coefficient for the effects between treatments (i = 1, 2; 1 = blood; 2 = virus), β_{3j} represents the coefficient for the effects between localities (j = 1, 2, 3; 1= Antonio Nariño; 2= Siloe; 3= Paso del Comercio), x_{1t} represents time, x_{2i} represents treatment, x_{3j} represents locality and ε_{tij} represents random error. In the model, interactions between times and localities, and between treatments and localities were also evaluated. The overall differences in gene expression over time of each gene among strains and localities were observed with expression ratios (R) estimated by exponentiating $2^{-\text{coefficient}}$. Coefficients comparing ΔCt values estimate the corresponding ΔΔCt values.

In both analyses, a two-sided significance level of 0.05 was used. All statistical analyses were completed in R Version 3.1.1 (R Core Team, 2014) and STATA/SE Version 12.0 (StataCorp LP, College Station, TX, U.S.A.).

Ethical statement

All protocols for mosquito feeding were approved by CIDEIM's Research Ethics Committee for Animal Experimentation.

Results

Vector competence for different DenV serotypes in selected strains

Viral load—Vector competence studies were carried out with the selected strains Cali-S (F_{31}) and Cali-MIB (F_{30}), which were 94 and 42% susceptible to DenV-2 infection, respectively. Ranges of virus concentration ($TCID_{50}$/mL) measured pre- and post-feeding for each serotype were: $10^{8.1}$–$10^{7.1}$ for DenV-1, $10^{8.1}$–$10^{7.0}$ for DenV-2, $10^{8.1}$–$10^{6.9}$ for DenV-3 and $10^{6.9}$–$10^{5.9}$ for DenV-4.

The results of Cali-S and Cali-MIB mosquitoes infected with the different DenV serotypes are shown in Fig. 1. Figure 1(A) shows the percentage of mosquitoes with each phenotype [S (susceptible), MEB (midgut escape barrier), MIB (midgut infection barrier)] observed in the susceptible strain (Cali-S). Figure 1(B) shows the percentage of mosquitoes with each phenotype observed in the refractory strain (Cali-MIB). The results show that Cali-S maintains a high susceptibility to all DenV serotypes, whereas Cali-MIB maintains a low susceptibility to all DenV serotypes. There were highly significant differences between the Cali-MIB (reference) and Cali-S strains for all DenV serotypes [strains: odds ratio (OR) 0.11, Z = 7.6, P < 0.001]. There were no significant differences in susceptibility within the Cali-S or Cali-MIB strains to the different dengue serotypes, using DenV-2 as the reference category (DenV-1: OR 1.21, Z = 0.63, P = 0.53; DenV-4: OR 1.39, Z = 1.07, P = 0.29). Significant differences were observed with DenV-3, but with higher and lower susceptibility maintaining the phenotype of the strains (DenV-3: OR 2.08, Z = 2.45, P = 0.01).

Vector competence and immune response analysis in mosquitoes collected from different localities

Vector competence for DenV-2 differed among the mosquitoes collected from the different localities around Cali (Fig. 2). Vector competence varied from 34% to 68% in the

susceptible phenotype and from 11% to 38% in the refractory MIB phenotype. The largest percentage of susceptible mosquitoes was found in the neighbourhood of Valle Grande (68%, $n = 62$), followed by Paso del Comercio (55%, $n = 44$), Siloe (52%, $n = 58$), and Mariano Ramos (52%, $n = 50$). The neighbourhood of Antonio Nariño had the highest percentage of refractory mosquitoes (38%, $n = 47$) (Fig. 2). Statistical differences in VC were found among collection sites ($\chi^2 = 15.65$, $P = 0.007$). Pairwise comparisons highlighted significant differences in VC between the following localities: Paso del Comercio and Navarro la Y ($\chi^2 = 6.63$, $P = 0.036$), Antonio Nariño and Navarro la Y ($\chi^2 = 6.59$, $P = 0.037$), and Antonio Nariño and Valle Grande ($\chi^2 = 14.71$, $P = 0.0006$). In the temporal gene expression analysis, mosquitoes from Paso del Comercio and Siloe were selected as being more susceptible, and mosquitoes from Antonio Nariño as being more refractory.

Temporal gene expression analyses

Differences in the expression of the selected genes between mosquitoes collected in Antonio Nariño (more refractory) and Paso del Comercio and Siloe (more susceptible) were measured, (Fig. 2). The expression levels of these genes in response to feeding on blood or blood + DenV-2 are shown in Fig. 3. With one exception, there were no statistically significant differences between gene expression levels in any of the mosquitoes at any time-point when all were fed on blood alone. When mosquitoes were exposed to DenV, higher levels of expression of *caspase 16*, *cathepsin-B* and *Aedronc* were observed, which were 20-fold higher at 24 h in mosquitoes collected from Antonio Nariño compared with mosquitoes from Paso del Comercio, and between four- and seven-fold higher than those in mosquitoes from Siloe (Fig. 3). With *Niemann–Pick Type C-2*, these differences in expression patterns were not observed between refractory Antonio Nariño and Paso del Comercio mosquitoes, but expression was six-fold higher in mosquitoes from Antonio Nariño than in those from Siloe.

The multiple linear regression model identified significant differences among collections from the various localities for *caspase 16* across the evaluated times compared with time 0 (24 h: $R = 0.45$, $t = 2.06$, $P = 0.042$; 36 h: $R = 0.33$, $t = 2.26$, $P = 0.007$; 48 h: $R = 0.33$, $t = 2.81$, $P = 0.006$), treatments (blood vs. blood + DenV) ($R = 1.95$, $t = -2.39$, $P = 0.019$), and additionally evidenced significant differences between Antonio Nariño and Siloe mosquitoes ($R = 0.31$, $t = 2.34$, $P = 0.02$). Significant differences were also observed with *Aedronc* among the evaluated times compared with time 0 (24 h: $R = 0.37$, $t = 2.15$, $P = 0.034$; 36 h: $R = 0.21$, $t = 3.41$, $P = 0.001$; 48 h: $R = 0.39$, $t = 2.04$, $P = 0.04$), but not among treatments. Significant differences were also observed for *Aedronc* between Antonio Nariño and Paso del Comercio mosquitoes ($R = 0.31$, $t = 2.02$, $P = 0.046$). No statistical differences were observed for *cathepsin-B* and *Niemann–Pick Type C-2* in either of the comparisons (data not shown).

Discussion

The selected *S. aegypti* strains, Cali-S and Cali-MIB, showed similar levels of VC for the four reference DenV serotypes, suggesting that the infection barrier in the refractory strain is

serotype-independent. Previous studies in *S. aegypti* on VC for different serotypes showed contrasting results: some studies showed similar levels of VC among serotypes (Cox *et al.*, 2011; Nguyen *et al.*, 2013), whereas other studies suggested a dependency on the virus isolate (i.e. local viral serotype adaptations) (Lambrechts *et al.*, 2009; Fansiri *et al.*, 2013). In the present study, the similar VC among serotypes in the selected strains supports their use as a model for understanding vector–virus interactions observed in field mosquitoes from Cali. The low VC variation observed within the selected strains may be attributable to their laboratory maintenance for over 30 generations.

Field-collected mosquitoes showed significant variability in their VC for DenV-2; susceptibility ranged from 68% (Valle Grande mosquitoes) to 34% (Antonio Nariño mosquitoes) as expected based on previous studies (Ocampo & Wesson, 2004).

Variability in VC has been observed at national (Dickson *et al.*, 2014), provincial (Carvalho-Leandro *et al.*, 2012) and municipal (Ocampo & Wesson, 2004) levels. These changes demonstrate the high variability in VC exhibited by different geographic populations of *S. aegypti*. It is likely that field *S. aegypti* with different levels of VC will emerge as changes in the genetic structure of vector populations influence their ability to transmit dengue (Sim *et al.*, 2013). Genetic studies on *S. aegypti* from Colombia suggest there are two overarching maternal lineages, the overall distribution of which has been influenced by microclimatic variables and reinvasion processes (Jaimes-Dueñez *et al.*, 2015). The process of reinvasion and gene flow across countries can significantly increase population-level genetic variation (Lima & Scarpassa, 2009) and hence influence DenV VC.

The variability in VC in field-collected Cali mosquitoes allowed the selection of susceptible and refractory populations from different localities in which to analyse gene expression. Mosquitoes from Antonio Nariño overexpressed apoptosis-related genes earlier in the infection, similar to the comparisons between Cali-S and Cali-MIB (Ocampo *et al.*, 2013). Expression of *caspase 16* and *Aedronc* showed significant differences over time and treatment. This was not the case for *cathepsin-B* and *Niemann–Pick Type C-2.* The few statistically significant differences in the multiple regression models between localities may be associated with genetic variability in the field-collected mosquitoes.

Apoptosis-related genes such as *caspase 16* and *Aedronc* may play important roles in the elimination of viruses. Apoptosis-mediated cell death has been reported previously in mosquito midguts and salivary glands in response to a wide range of arboviruses, and these mechanisms may be associated with decreased susceptibility (Vaidyanathan & Scott, 2006; Kelly *et al.*, 2012; Ocampo *et al.*, 2013).

Cathepsin-B is a cysteine protease produced by the fat body in response to blood feeding (Cho *et al.*, 1999) and is secreted into the haemolymph during vitellogenesis (Price *et al.*, 2011). *Cathepsin-B* has previously been associated with the regulation of cell death (Stoka *et al.*, 2001). Although an overexpression of *cathepsin-B* at 24 h in mosquitoes from Antonio Nariño compared with both susceptible localities was observed (Fig. 3), the regression analysis does not show significant differences.

Niemann–Pick Type C-2 is a soluble protein with a lipid domain that appears to be important for pathogen recognition. Silencing this gene produced an increase in midgut resistance to DenV, suggesting that this gene plays a role as a DenV agonist (Jupatanakul *et al.*, 2014). Niemann–Pick Type C-2 is a protein homologous to MD2 in mammals, and is a receptor required for activation of the Toll pathway (Dong *et al.*, 2006; Sim *et al.*, 2012).

In the present study, standard IIF was used as the reference standard method to determine the number of mosquitoes that have disseminated DenV (presence or absence of the virus) as a predictor of VC rather than of the intensity of infection. However, future studies could compare quantitative DenV viral loads with gene expression to elucidate correlations between levels of expression of apoptosis-related genes and VC in susceptible mosquitoes.

These results suggest that the overexpression of apoptosis-related genes observed in the Cali-MIB strain arose in field mosquitoes, is maintained in selected laboratory strains, and may contribute to the refractoriness of Cali-MIB to all four DenV serotypes. Evaluating the VC of Cali-MIB to other arboviruses, such as the Zika and Chikungunya viruses, will determine whether this is a general antiviral response, a response to flaviviruses, or a specific response to DenV. This study validates the use of the Cali-S and Cali-MIB strains as providing an appropriate model in which to study vector–DenV interactions, and corroborates the involvement of apoptosis as a viral elimination mechanism.

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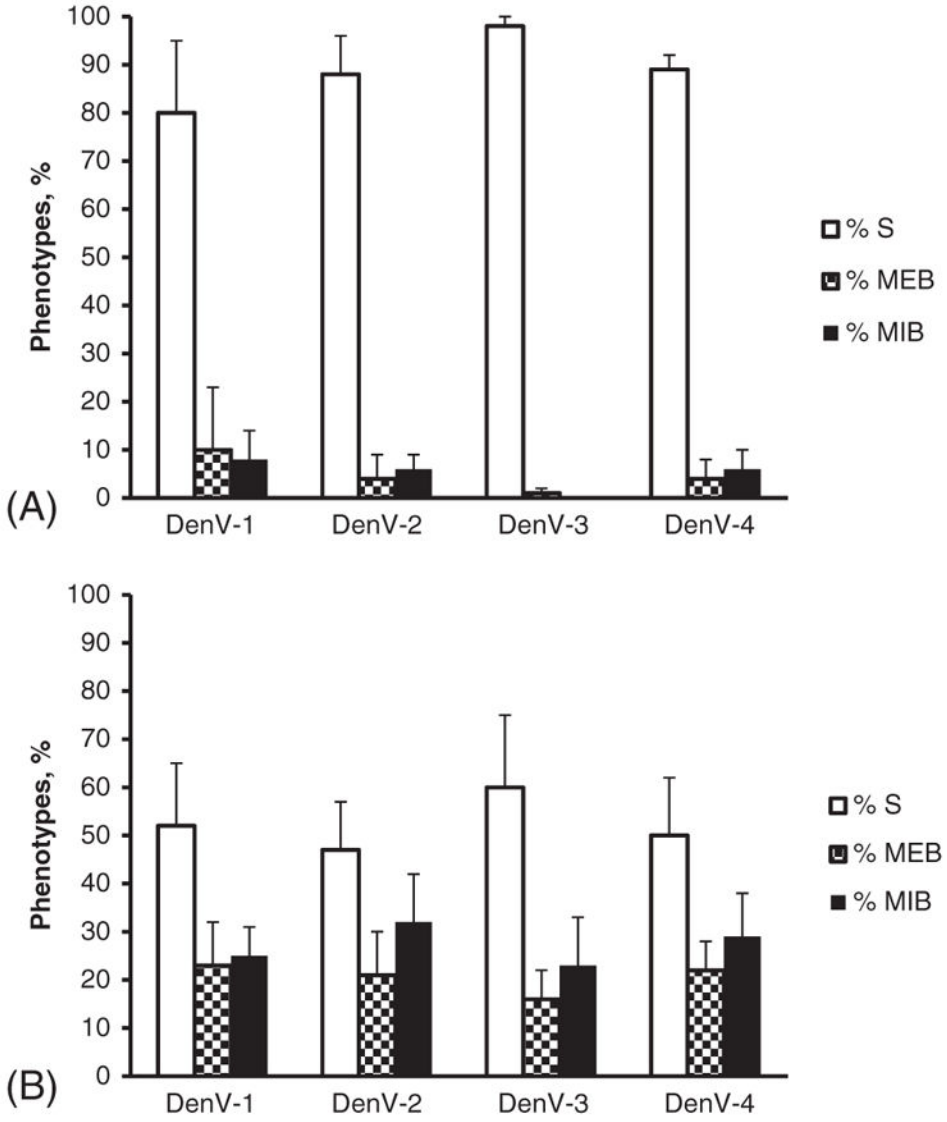

Fig. 1.

(A) Mean ± standard deviation percentages of mosquitoes with each phenotype after infection with DenV-1, DenV-2, DenV-3 and DenV-4 in (A) the susceptible strain (Cali-S) and (B) the refractory strain (Cali-MIB). DenV, dengue virus; S, susceptible; MEB, midgut escape barrier; MIB, midgut infection barrier.

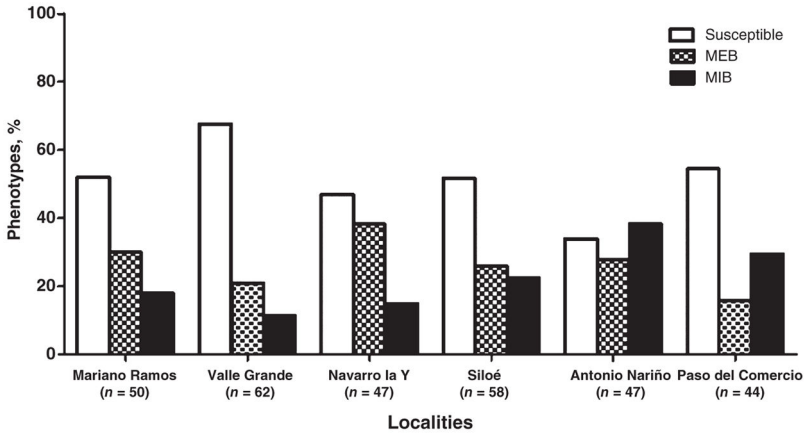

Fig. 2.

Vector competence for dengue virus serotype 2 in colonized mosquitoes from each field collection site, showing percentages of mosquitoes observed with each phenotype. MEB, midgut escape barrier; MIB, midgut infection barrier.

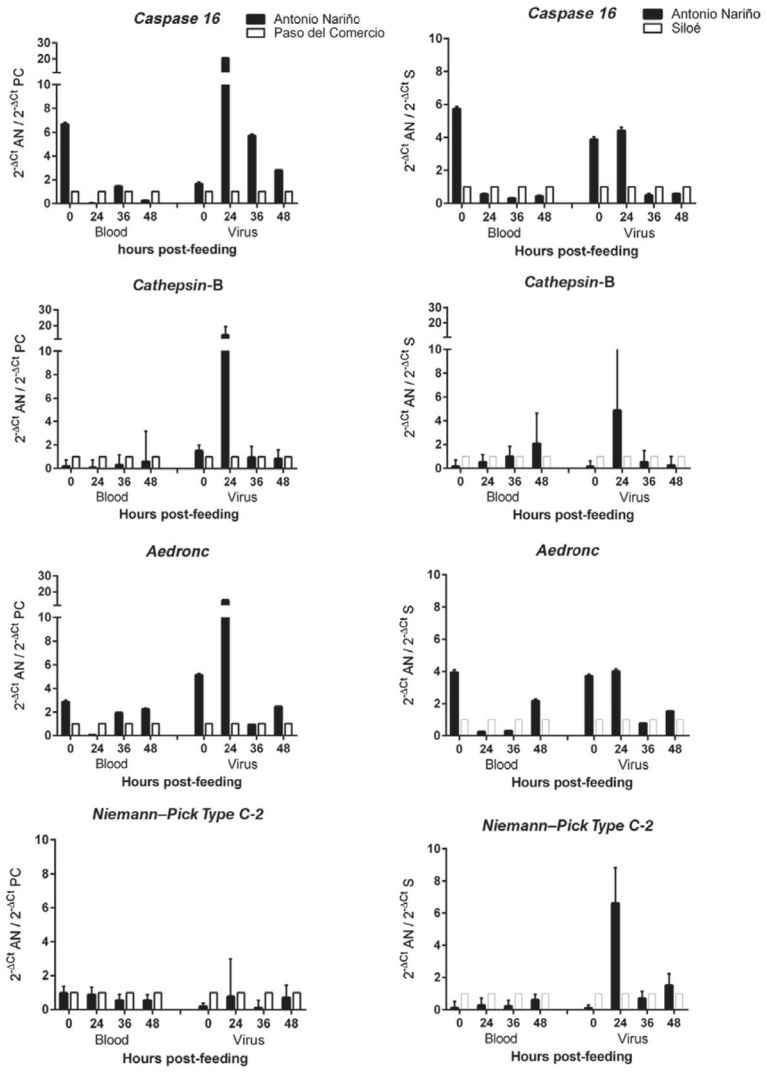

Fig. 3.

Comparisons of midgut gene expression of *caspase 16*, *cathepsin-B*, *Aedronc* and *Niemann–Pick Type C-2* between mosquitoes from Antonio Nariño (AN) and Paso del Comercio (PC) (left column) or Siloe (S) (right column) after feeding on blood or dengue virus-infected blood. In each pairwise comparison per time-point, expression levels in the Paso del Comercio or Siloe mosquitoes were arbitrarily set at 1, and expression levels in the Antonio Nariño mosquitoes represent relative fold-differences in expression.

Table 1

Primers used in quantitative polymerase chain reactions.

Target gene	Transcript ID	Sequence	Temperature
Caspase 16	AAEL005956-RA	5′-TCCGCTATCTTCATATTGTATCCTTTG-3′	60 °C
Aedronc	AAEL011562–RA	5′-CAACTTTCCAACTGCCTATAAATTGC-3′ 3′-CTCCACCGTATCGTTATTGTTCTTAG-5′	60 °C
Cathepsin-B	AAEL007585-RA	5′-CAAAGCACTCCCTTCCATC-3′ 3′-CACGAGCGTCGAATGTATC-5′	57 °C
Niemann–Pick Type C-2	AAEL015136-RA	5′-GCACTCGTCCCAGCTGTAATG-3′ 3′-CACTGACCAGCGGATAGATGG-5′	57 °C
β-Actin	AAEL001928-RA	5′-AAGGCTAACCGTGAGAAGATGAC-3′ 3′-GATTGGGACAGTGTGGGAGAC-5′	60 °C