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Inhibition of *defensin* and *cecropin* responses to dengue virus 1 infection in *Aedes aegypti*

Inhibición de las respuestas de *defensina* y *cecropina* contra la infección del virus dengue 1 en *Aedes aegypti*

Dengue virus 1 infection in *Aedes aegypti*

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Author's contribution:

Yda Méndez: accomplished the experiments

César Pacheco: designed the figures and supplementary material.

Flor Herrera: conceived the study, was in charge of overall direction and wrote the manuscript.

All authors discussed the results and contributed to the final manuscript.

Introduction. It is essential to determine the interactions between viruses and mosquitoes to diminish dengue viral transmission. These interactions constitute a very complex system of highly regulated pathways called the Innate Immune System of mosquito that produces effector molecules like antimicrobial peptides (AMP). These AMP function against bacterial and fungal infections, but on virus infections, there is less information.

Objective. To determine the expression of two AMP genes (*Defensin A* and *Cecropin A* genes) in *Aedes aegypti* mosquitoes infected with DENV-1.

Materials and methods. The F1 generation of mosquitoes orally infected with DENV-1 was used. Infection assays in conjunction with real-time PCR analysis were used to determine whether the *Defensin A* and *Cecropin A* genes have potential roles in controlling DENV-1 replication in *Ae. aegypti*. As a reference, similar experiments were performed with bacteria *Escherichia coli*

Results. Basal levels of *defensin A* and *cecropin A* mRNAs were expressed in uninfected mosquitoes at different times post-blood feeding. The infected mosquitoes experienced reduced expression of these mRNAs by at least eightfold when compared to uninfected control mosquitoes at all time post-infection. In contrast with the behavior of DENV-1, results show that bacterial infection produced up-regulation of *Defensin* and *Cecropin* genes; however, the induction of transcripts occurred at later times (15 days).

Conclusion. DENV-1 virus inhibits the expression of *Defensin A* and *Cecropin A* genes of wild *Ae. aegypti* population from Venezuela.

Key words: *Aedes aegypti*; dengue virus; alpha-defensins; cecropins; *Escherichia coli.*

Introducción: Es esencial determinar las interacciones entre los virus y los mosquitos para disminuir la transmisión viral. Estas interacciones constituyen un sistema muy complejo y muy regulado, llamado el Sistema Immune Innato del mosquito. Este sistema produce moléculas efectoras como los péptidos antimicrobianos (AMP) los cuales funcionan contra las infecciones bacterianas y fúngicas, pero se tiene poca información de su acción sobre los virus.

Objetivo: Determinar la expresión de dos genes AMP (genes *Defensina A* y *Cecropina A)* en mosquitos *Aedes aegypti* infectados con DENV-1.

Materiales y métodos: Se infectaron mosquitos de generación F1, oralmente con DENV-1. Los ensayos de infección junto con análisis de PCR en tiempo real se usaron para determinar si los genes *Defensina A* y *Cecropina A* tienen un papel potencial en el control de la replicación de DENV-1 en *Ae. Aegypti*. Similarmente, como referencia, se infectaron mosquitos con bacteria *Escherichia coli*.

Resultados: Los mosquitos no infectados expresaron niveles basales de los ARNm de *defensina A* y *cecropina A*, después de la alimentación a tiempos diferentes. Los mosquitos infectados experimentaron una reducción en la expresión de estos ARNm, al menos ocho veces con respecto a los mosquitos control, en todos los tiempos después de la alimentación. En contraste con el comportamiento del virus DENV-1, los resultados mostraron que la infección bacteriana produjo una regulación positiva de los genes *Defensina* y *Cecropina*; sin embargo, la inducción de los transcritos ocurrió a tiempos tardíos (15 días).

Conclusión: El virus DENV-1 inhibe la expresión de los genes *Defensina A* y *Cecropina A* de una población silvestre de Venezuela.

Palabras clave: *Aedes aegypti*; virus del dengue; alfa-defensinas; cecropinas; *Escherichia coli*.

In Venezuela, dengue is the most important arboviral disease affecting humans and its incidence and prevalence are rising annually (1). Until now, there is not a vaccine to avoid DENV infections; therefore, the vector control is the only way to restrain these disease risks (2).

The arboviruses, like DENV, have to experience a series of critical steps that demand their interplay with different tissues and it last for days or weeks until transmission can occur (2-4). These tissues represent barriers that restrict virus growth through, among others, immune molecules with antipathogenic activity that belong to a very complex system of highly regulated pathways called the innate immune system of mosquitos. Toll, IMD, JAK/STAT and RNAi are the primary immune signaling pathways (2-5).

Different strategies to diminish viral transmission like the use of genetically engineered vectors as well as natural symbionts such as *Wolbachia* have been considered (6,7). In any strategy to be used for controlling dengue transmission, it is essential to determine the interactions between viruses and mosquitoes, in particular, their innate immune system.

Toll and IMD pathways produce effector molecules, called antimicrobial peptides (AMPs), low molecular weight proteins, that have been well known to function against bacterial and fungal infections, but less information is disclosed on virus infections. Reports suggest that dengue virus infection is controlled by the Toll pathway in mosquitoes (8), that Toll and IMD pathways are upregulated in the Sindbis virus that infected mosquitoes (9), and DENV-2 (10,11). Contrary results have been found, such as the inhibition of Toll's innate immune response in the

salivary glands by DENV-2 with 3'UTR substitutions associated with high epidemiological fitness and enhanced production of infectious saliva (12).

In our study, it was found that the expression of *Defensin A* and *Cecropin A* genes, two AMP genes mediated by the Toll pathway, was significantly decreased in *Ae. aegypti* mosquitoes infected with DENV-1 suggesting that the infection progresses by suppressing the Toll pathway.

Materials and methods

Mosquito collection

Ae. Aegypti mosquitoes were collected as larvae from Maracay City, Venezuela and the F1 generation were obtained.

Dengue virus, bacteria and infection processes

Infections were performed using one DENV-1 isolate (LAR23644) which was isolated from a patient in Maracay in 2007. Viruses were serially passaged in *Ae. Albopictus* C6/36 cells, infected supernatants harvested, titered via plaque forming assay, and then frozen at -80 °C. Viral titre was $4{,}8x10^5$ PFU/mL. For oral infection experiments, viral stocks were mixed 1:1 with human red blood cells called "HRBC" (PBS washed) and fed to mosquitoes (sugar starved 24 h) via membrane feeders. Groups of mosquitoes were also fed on HRBC only. Immediately post-feeding, fully engorged specimens were transferred to new cages, held under standard rearing conditions and provided sucrose.

At different times after feeding, ≈30 mosquitoes per each time were collected. It was used early times (5 & 24 hours) to see if the virus was inactivated by some mosquito

antiviral defense mechanism present in the mosquito´s gut and late times (10 & 15 days) to detect viral replication in the mosquito´s body

To determine the percentage of infection, dissemination and potential transmission of the virus in the vector, 50 individual mosquitoes (fed with the virus similarly as before, and collected in 15 days), were dissected in the abdomen (infection), legs and wings (dissemination) and salivary glands (potential transmission). The virus found in this last tissue has the potential to be transmitted, because the only way to measure transmission is analyzing the saliva of the mosquitoes (13). Prior to the start of their lysis, all the tissues were washed three times with 200 µL of PBS to discard any contamination. Mosquitoes were stored at -80 °C.

Similar infections were carried out with *E. coli* bacteria that were cultured to OD_{600} 0.8, pelleted, washed and resuspended on PBS. The bacteria culture was mixed with HRBC in equal proportions and the previous methodological procedure used for viral infection was also applied.

Detection and typing of dengue viruses in Ae. aegypti

RNA extraction, detection and typing of dengue viruses in pools of whole bodies or in dissected samples of *Ae. Aegypti* were performed, according to Urdaneta et al (14).

Quantitative RT-PCR* (qPCR) for *measuring gene expression

Gene expression was determined by relative quantification, relating the qPCR signal of the *defensin A* or *cecropin A* gene transcript in a mosquito group fed on virus or bacteria mixed with HRBC to that of a control group (calibrator) fed only on HRBC. qPCR was conducted in a reaction volume of 25 µL in a 96-well plate

containing 0.5 µg of template based on the initial RNA concentration, and 200 nM forward and reverse primers, using Go *Taq* qPCR real time (Promega Corporation, USA), on 7500 Real-Time PCR System (Applied Biosystems, Massachusetts, USA) using the program: 2 minutes of preincubation at 95 ºC followed by 40 cycles for 30 s at 95 ºC and one minute at 60 ºC. Designed specific primers used were: *Defensin A* gene (sense: 5'-AACTGCCGGAGGAAACCTAT-3'; antisense: 5'-TCTTGGAGTTGCAGTAACCT-3') and *Cecropin A* gene (Sense: 5'-CGAAGTTATTTCTCCTGATCG-3'; antisense: 5'-AGCTACAACAGGAAGAGCC-3'). To normalize the data, the *α-Tubulin* gene (Sense: 5'-GCGTGAATGTATCTCCGTGC-3'; antisense: 5'-AGCTACAACAGGAAGAGCC-3') was used as an endogenous reference.

α-tubulin, defensin A and *cecropin A* primer pairs were assessed and for each one it was found: a) the observed efficiency was near to 100% (Supplementary file 1), the amplification specificity was displayed through the production of a unique peak in melt-curve analysis (Supplementary file 2) and it was corroborated by sequencing the PCR products from each gene in both directions using the PCR primers (data not shown). Sequencing reactions were performed with ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit on an Applied Biosystem Model ABI 3130XL. Therefore, the $2^{-\Delta\Delta Ct}$ method of relative quantification was used to appraise relative gene expression.

Control and virus infected pool samples (≈30 mosquitoes/pool) for different times after feeding (5 h, 24 h, 10 days and 15 days) were used in the qPCR reaction (a total of 8 pools: ≈240 mosquitoes). The control values were very close at all times,

therefore the average of all them was taken as the calibrator. Each qPCR experiment was repeated three times with three replicates of each one. Similar experiments were carried out with the bacteria *E. coli.* The average and standard deviation (SD) of the C_Ts from the three replicates were determined and the average was only approved if the SD was < 0.38 (15). Repeatability and reproducibility for the assay were calculated by a percent coefficient of variance (%CV) within and between assays (Supplementary file 3).

N-fold copy numbers of the *Ae. aegypti defensin A* and *cecropin A* gene transcripts relative to the control in each assay were calculated using geometric means among the three experiments.

Ethical approval. Not required.

Results

Stability and Replication of DENV-1

Determining whether the DENV-1 was stable at early (5 and 24 hours) and replicated at late time (10 and 15 days) post-infection (d.p.i.) in the mosquito, RT-PCR amplification followed by agarose gel electrophoresis analysis of the products was performed. Fig. 1 revealed the presence of DENV-1, with the position of the cDNA band at 482 bp, at all time points studied. The replication has been further corroborated since in dissected samples of 50 individual mosquitoes, they attained 70%, and 100% of viral infection and dissemination efficiency by 15 days post-infection. With respect to the virus present in the salivary glands, it also demonstrated replication (45%) and potential transmission efficiency (Supplementary file 4).

Inhibition of defensin and cecropin mRNAs by DENV-1

The relative expression levels of *Defensin A* and *Cecropin A* genes in DENV-1-infected *Ae. aegypti* mosquitoes as compared to the calibrator are presented in Fig. 2A. It shows that both mRNAs were detectable in control mosquitoes; however, a significant decrease in abundance took place at all time points measured, observing at least 5- to 8-fold less amounts of *defensin* and *cecropin* mRNAs, respectively, in mosquitoes infected with DENV-1.

Induction of defensin and cecropin mRNAs by bacteria

The response of the field population of *Ae. aegypti* mosquitoes to bacteria was contrary to the previously described virus response because bacterial infection as expected did not produce down-regulation of both genes (Fig. 2B); however, the induction of transcripts occurred at later times (15 days).

Discussion

Exists discrepancy in the immune system´s reaction from mosquitoes in the presence of DENV-1. The response could be stimulation (2,8,10,11), or suppression *in vivo* (9,16-18), and *in vitro* (19, 20). This discrepancy may depend on the viral strain used, the genetic history of the vector and the mode of transmission (3,21). We found decreased expression of *Defensin A* and *Cecropin A* genes using the F1 generation of wild mosquitoes infected with DENV-1. Similar results were reported with DENV-2-infected field *Ae. aegypti* populations (6).

The specific molecular mechanism by which DENV act remains uncharacterized. The virus may be able to knock down the expression of some factor needed to induce the expression of *defensin* and *cecropin* mRNAs similar to the role reported

for a protein in *Ae. aegypti*, *Ae*FaDD (22). Alternatively, the DENV may directly target and inhibit transcription of both genes.

The suppression of the innate immune responses of mosquitoes found in this study was time-independent contrary to other reports (using similar times as this work: 1, 2, 7 and 14 days) (17) which implies that the DENV may exert continuous or for some period of time immunomodulatory activity in mosquitoes. This is critical for defining vector competence of local mosquitoes, as well as dengue transmission intensity in a particular area.

Bacterial infection as expected did not produce down-regulation of *Defensin* and *Cecropin* genes (23,24); however, the induction of the transcripts occurred at later times (Fig. 2B). These data could indicate that the capacity of this wild *Ae. Aegypti* mosquitoes to mount a highly effective production of *defensin* and *cecropin* to control invading bacteria would take some time, which may be needed to inactivate bacterial growth factors.

In conclusion, DENV-1 inhibits the expression of *Defensin A* and *Cecropin A* genes of wild *Ae. aegypti* population from the Maracay city in Venezuela. Therefore, how the virus participates in this inhibitory mechanism and what are the viral effector molecules are important questions to be answered.

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Conflict of interests

None declared

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Figure 1. Detection of DEN-1 in *Ae. aegypti* by gel electrophoresis on a 2% agarose gel. DNA amplicons generated by RT-PCR of the RNA extracted from dengue viruses in *Ae. aegypti* at different times post infection. Lanes 1-4: 5h, 24h, 10 days and 15 days respectively; Lane 5, Negative Control; Lane 6, Positive Control of DEN-1 with a product size of 482 bp. Lane M, 100 bp ladder marker. DNA sizes are given in base pairs.

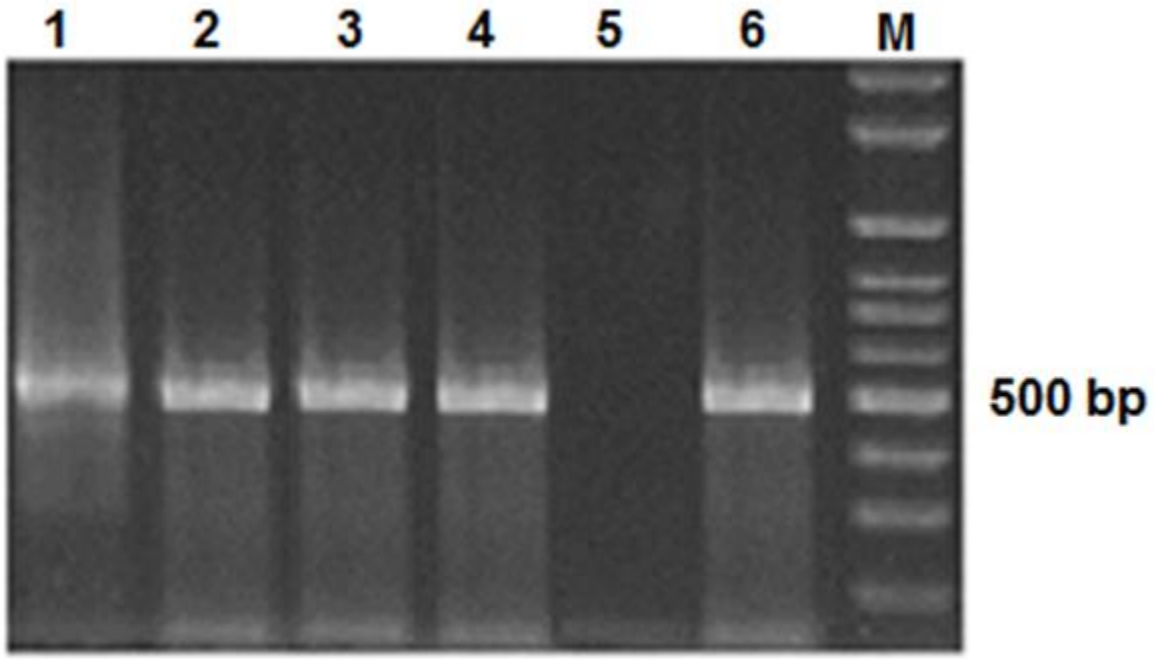
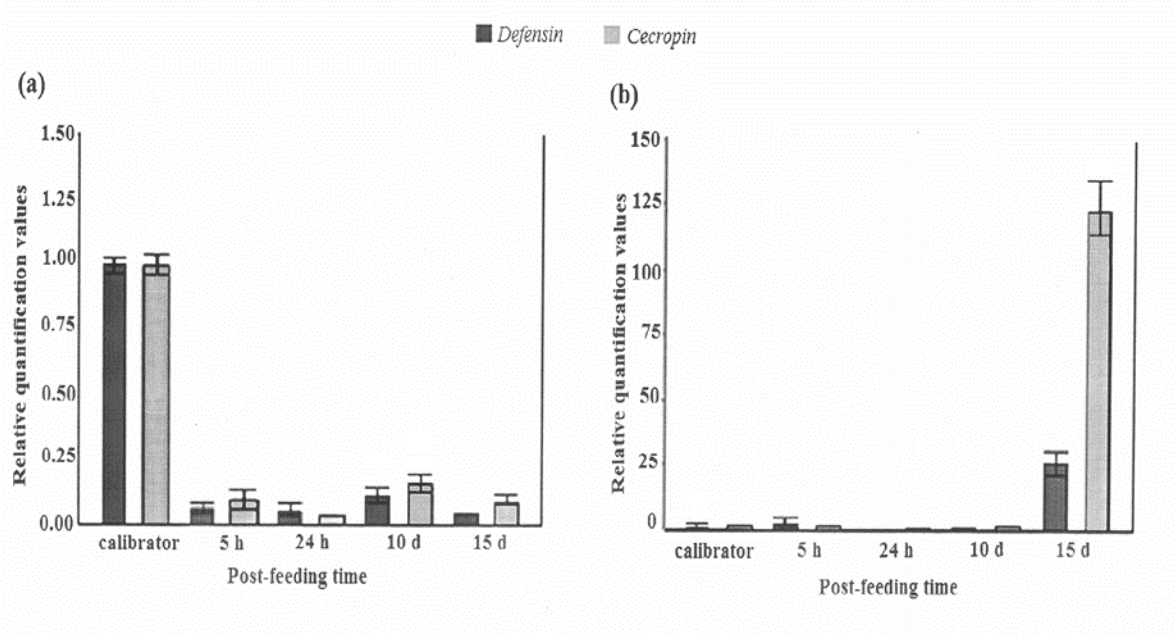
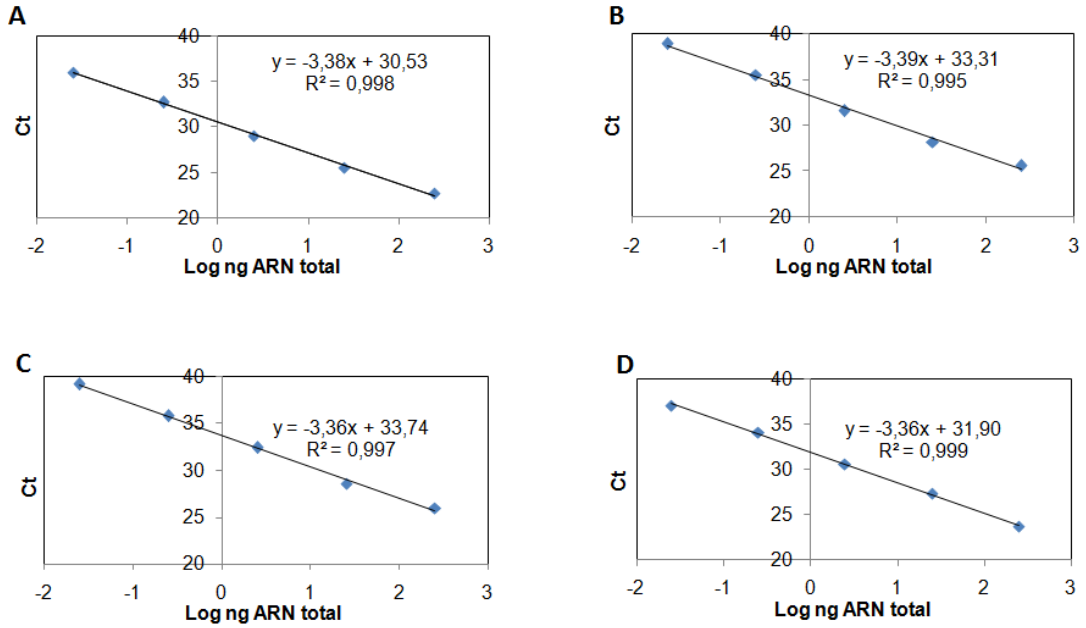


Figure 2. Comparison of immune responses to DENV-1 and *E. coli* bacteria in field-collected *Ae. aegypti.* Averaged data from three independent real-time qPCR experiments wereused to assess the expression of each of the selected immune genes in the *Ae. aegypti* mosquitoes infected with the DEN-1 virus (a) or *E. coli* bacteria (b), with the host *α- Tubulin* as an internal reference control normalizes the data. For each pathogen, the control values for both genes at all the time points were very similar. In every case, the average of all these values was used as the calibrator. The $2^{-\Delta\Delta CT}$ method was used to calculate fold change for each gene.

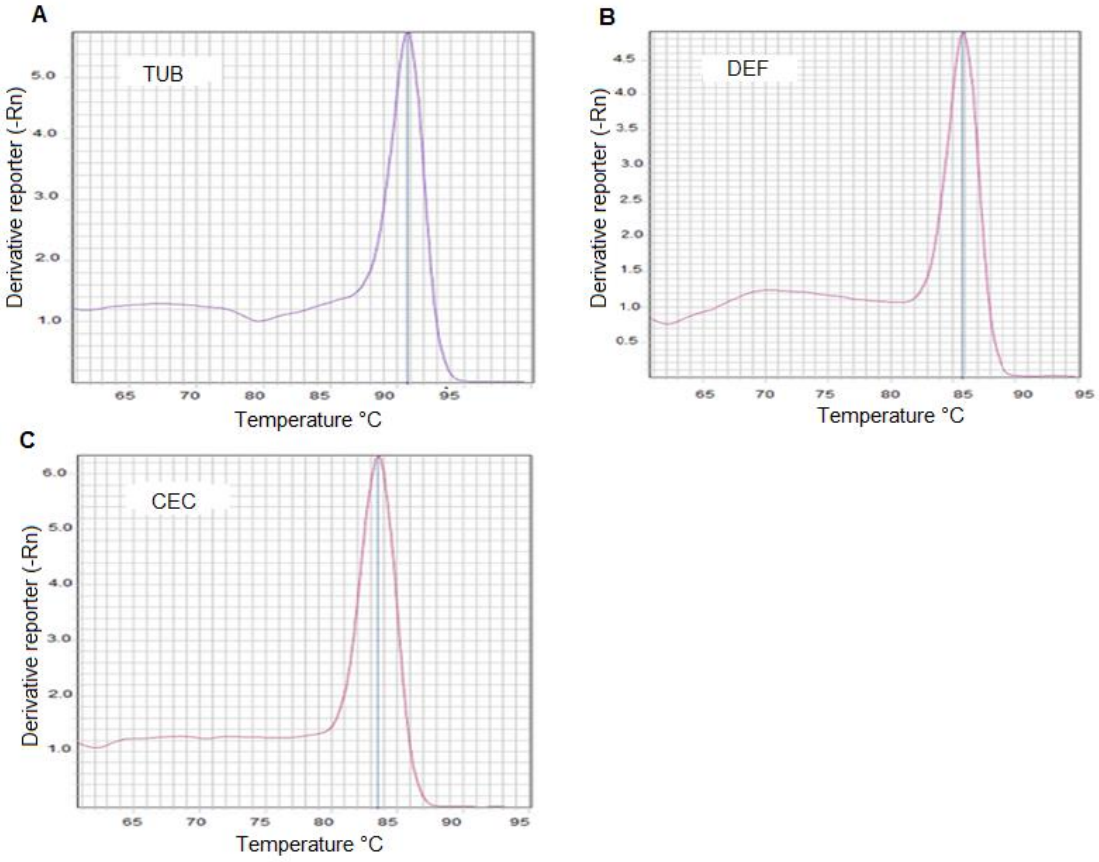


Supplementary file 1. Efficiency of gene amplification. The qPCR amplification efficiencies of *α-tubulin, defensin A* and *cecropin A* genes. DNAs of *each gene* was diluted in serial 10-fold ranges and the Ct value at each dilution was measured. The Ct represents an average of triplicate in two independent experiments. Then, a curve was obtained for a) *α-tubulin,* b) *defensin A* or c) *cecropin A* gene from which qPCR efficiencies (E) was assessed. The slopes of the curves were used to calculate E, according to the equation: $E = 10^{(-1/slope)} - 1 \times 100$, where E = 100 corresponds to 100% efficiency.



The efficiencies of amplification obtained for the genes after applying the equation were:
A) α-tubulin gene, 97.3%
B) defensin A gene 97.2%
C) cecropin A gene 98.5%

Supplementary file 2. Melting Curve. DNA Melting Curve Analysis qPCR for Detection of the genes' specificity. Three fragments the first from the *α-Tubulin* gene, the second from the *Defensin A* and the last from the *Cecropin A* gene were synthesized by the qPCR using specific primers for each gene. The resulting products were subjected to post-PCR melt analysis. There was the detection of only one peak with primers for A) *α-Tubulin* gene B) *Defensin A* gene or C) *Cecropin A* gene



Supplementary file 3. Repeatability and reproducibility of qPCR assay

Table I. Repeatability of qPCR assay for *Defensin* A gene in DEN 1-*infected Ae. aegypti mosquitoes*

Sample	Replicates of Run (Ct)			mean	SD	Repeatability (CV)	% CV
	1	2	3				
Calibrator	25.6	26.8	27.1	26.5	0.8	0.03	3.0
5 h	31.5	32.4	31.6	31.8	0.5	0.02	1.5
24 h	30.5	31.2	30.6	30.8	0.4	0.01	1.2
10 d	31.8	31.5	31.8	31.7	0.2	0.01	0.5
15 d	32.6	31.3	32.6	32.2	0.8	0.02	2.3

Table II. Repeatability of qPCR assay for *Cecropin A* gene in DEN 1-*infected Ae. aegypti mosquitoes*

Sample	Replicates of Run (Ct)			mean	SD	Repeatability (CV)	% CV
	1	2	3				
Calibrator	23	24	22	23.0	1.0	0.04	4.3
5 h	26.9	27.5	27.4	27.3	0.3	0.01	1.2
24 h	29.2	29.5	29.2	29.3	0.2	0.01	0.6
10 d	26.9	26.7	26.4	26.7	0.3	0.01	0.9
15 d	28.7	28.8	28.8	28.8	0.1	0.00	0.2

Table III. Repeatability of qPCR assay for *Defensin A* gene in *E. coli-infected Ae. aegypti mosquitoes*

Sample	Replicates of Run (Ct)			mean	SD	Repeatability (CV)	% CV
	1	2	3				
Calibrator	27	27.2	26.8	27.0	0.2	0.01	0.7
5 h	25.4	25.7	25.4	25.5	0.2	0.01	0.7
24 h	27.9	27.1	27.5	27.5	0.4	0.01	1.5
10 d	25.9	26.9	26.6	26.5	0.5	0.02	1.9
15 d	22.8	23.1	22.8	22.9	0.2	0.01	0.8

Table IV. Repeatability of qPCR assay for *Cecropin A* gene in *E. coli-infected Ae. aegypti mosquitoes*

Sample	Replicates of Run (Ct)			mean	SD	Repeatability (CV)	% CV
	1	2	3				
Calibrator	26	26	25	25.7	0.6	0.02	2.2
5 h	24	25	24	24.3	0.6	0.02	2.4
24 h	25.1	25.9	25.8	25.6	0.4	0.02	1.7
10 d	24.8	25.3	25.3	25.1	0.3	0.01	1.1
15 d	23.9	24	24.4	24.2	0.4	0.01	1.5

For all tables, the repeatability was calculated as the percent coefficient of variance (%CV) of Ct triplicate values of a sample within a single experiment.

Table V. Reproducibility of qPCR assay for *Defensin* A gene in DEN 1-infected *Ae. aegypti* mosquitoes

Sample	Run (%CV)			mean	SD	*Reproducibility Run 1 ~ Run 2 ~ Run 3 (% CV)
	1	2	3			
Calibrator	3.0	2.8	2.9	2.9	0.10	0.034
5 h	1.5	1.4	1.5	1.5	0.08	0.051
24 h	1.2	1.4	1.2	1.3	0.11	0.084
10 d	0.5	0.4	0.5	0.5	0.08	0.155
15 d	2.3	2.2	2.3	2.3	0.07	0.030

Table VI. Reproducibility of qPCR assay for *Cecropin A* gene in DEN 1-infected *Ae. aegypti* mosquitoes

Sample	Run (%CV)			mean	SD	*Reproducibility Run 1 ~ Run 2 ~ Run 3 (% CV)
	1	2	3			
Calibrator	4.3	4.3	4.2	4.3	0.08	0.018
5 h	1.2	1.3	1.3	1.3	0.07	0.055
24 h	0.6	0.5	0.6	0.6	0.06	0.098
10 d	0.9	0.8	0.8	0.8	0.08	0.098
15 d	0.2	0.2	0.1	0.2	0.06	0.347

Table VII. Reproducibility of qPCR assay for *Defensin A* gene in *E. coli*-infected *Ae. aegypti* mosquitoes

Sample	Run (%CV)			mean	SD	*Reproducibility Run 1 ~ Run 2 ~ Run 3 (% CV)
	1	2	3			
Calibrator	0.7	0.9	0.7	0.8	0.11	0.135
5 h	0.7	0.8	0.7	0.7	0.06	0.089
24 h	1.5	1.4	1.6	1.5	0.10	0.070
10 d	1.9	1.9	1.8	1.9	0.07	0.038
15 d	0.8	0.9	0.8	0.8	0.07	0.090

Table VIII. Reproducibility of qPCR assay for *Cecropin A* gene in *E. coli*-infected *Ae. aegypti* mosquitoes

Sample	Run (%CV)			mean	SD	*Reproducibility Run 1 ~ Run 2 ~ Run 3 (% CV)
	1	2	3			
Calibrator	2.2	2.1	2.2	2.2	0.08	0.035
5 h	2.4	2.6	2.4	2.5	0.12	0.051
24 h	1.7	1.7	1.6	1.7	0.06	0.035
10 d	1.1	1.2	1.2	1.2	0.03	0.025
15 d	1.5	1.3	1.5	1.4	0.11	0.075

Supplementary file 3. Infection, Dissemination and Potential Transmission of DENV-1 in *Aedes aegypti* mosquitoes

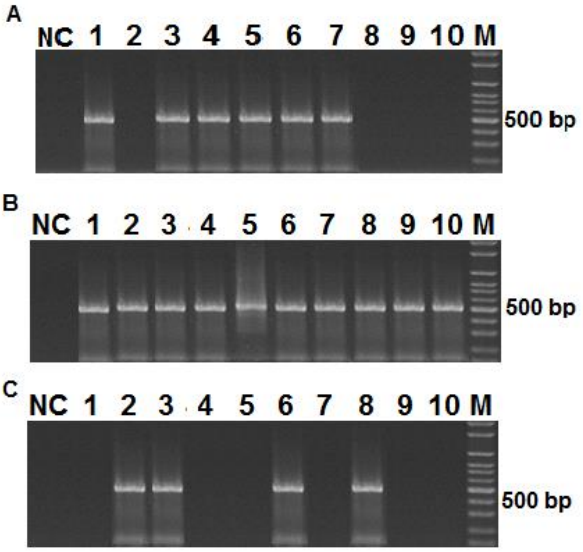


Figure. DENV-1 detection in *Ae. aegypti*. Mosquitoes (50) were infected with DENV-1 (1.6 x10^5 PFU/mL) and after 15 days the RNAs were extracted from different tissues of individual mosquitoes. RNAs were visualized using nested PCR followed by electrophoresis on a 2% agarose gel and SYBR Green staining. The 482 bp band corresponding to the DENV-1 amplification product was shown in abdomens (A), wings and legs (B) and heads (C).

Table. Rates of Infection, Dissemination and Potential Transmission of the DENV-1 in *Ae. aegypti* mosquitoes

Infection (%) [a]	Dissemination (%) [b]	P. Transmission (%) [c]	
70 (35/50)	100 (35/35)	45.7 (16/35)	(p<0,01)*

[a] Rate of infection: Number with DEN virus- positive abdomens/number tested
[b] Rate of Dissemination: Number with DEN virus- positive legs and wings/number with DEN virus-positive abdomens
[c] Rate of Potential Transmission: Number with DEN virus- positive heads/number with DEN virus-positive abdomens
*Open Epi Versión 3.03 (**Dean AG, Sullivan KM, Soe MM. OpenEpi: Open Source Epidemiologic Statistics for Public Health, Versión. www.OpenEpi.com, updated 2013/04/06, accessed 2020/04/03)** was used for statistical analysis