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First Evidence of Horizontal Transmission by Fecal Shedding of Dengue Virus 4 Among *Aedes aegypti* Larvae (Diptera: Culicidae) Under Laboratory Conditions

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Abstract

The transmission pathways of dengue virus (DENV) among mosquitoes are a topic that has gained relevance in recent years because they could explain the maintenance of the virus in the wild independently of the human–mosquito horizontal transmission cycle. In this regard, *Aedes aegypti* larvae exposed to supernatants of C6/36 cells infected with DENV-4 were evaluated for virus excretion in feces and viability of infection in immature stages (larvae). The results demonstrate that larvae excrete DENV-4 in their feces with the potential to at least infect immature individuals of the same species. A horizontal transmission pathway of larvae–larvae DENV-4 under laboratory conditions is suggested.

Keywords: dengue virus, viral excretion, horizontal transmission, *Aedes aegypti*

Introduction

Two routes of transmission for dengue virus (DENV) among *Aedes aegypti* have been described: vertical (VT) and sexual (ST) (Apodaca-Medina et al. 2018, Sánchez-Vargas et al. 2018). It is believed that these infection mechanisms contribute to the maintenance of the virus in wild mosquito populations independently of viremia in humans (Sánchez-Vargas et al. 2018). Another possible mechanism for DENV amplification could be occurring within immature mosquito populations. The susceptibility of *Ae. aegypti* to DENV has been reported when exposed to infected tissue or viral suspensions under controlled conditions (Bara et al. 2013).

In a hypothetical scenario of natural infection, this could occur by cannibalism of VT-infected larvae or even by exposure to viable viral particles excreted in feces of immature stages. Although it has not been proven that *Ae. aegypti* larvae excrete DENV particles, this phenomenon occurs in adults (Fontaine et al. 2016), which suggests the possibility of this also occurring in larvae of *Ae. aegypti* larvae. The objective of this study was to test, under laboratory conditions, the excretion of DENV in larval feces and its potential to infect *Ae. aegypti* larvae.

Materials and Methods

This study was approved by the Biosafety and Bioethics Committee Postgraduate in Biological Sciences, Faculty of Biology, Autonomous University of Sinaloa.

Sixteen groups of 30 *Ae. aegypti* larvae (New Orleans Strain: after the L-2 stage) were exposed to 70 and 140 μL (8 groups per volume) of supernatant of DENV-4 infected C6/36 cells placed in plastic receptacles containing 15 mL of water (temperature 23°C ± 2°C). At 24 h postexposure, the larvae were washed with water and placed in new containers. Approximately 600 μL of water with feces per group were

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collected at 24, 48, and 120 h postwashing. The feces were homogenized with a sterile tip together with the collected water and centrifuged at 14,000 *g*/5 min. Two supernatants from each larval infection assay (volume 70 and 140 μL) were filtered using a 0.2 μm filter.

From the previously filtered supernatants, the infective viability of DENV-4 was determined by cytopathic effect in an *in vitro* assay using the RAW 264.7 cell line and by molecular detection in *Toxorhynchites* sp. Regarding the first evaluation in a 24-well microplate, 50,000 cells were seeded and incubated at 37°C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% of fetal bovine serum at 10%, penicillin 1% and, streptomycin, until obtained a confluence of 85–95%. A volume of 100 μL of supernatant in 900 μL of DMEM were inoculated. The wells were monitored every 24 h until the cytopathic effect was observed.

Subsequently, the supernatants were inoculated into *Toxorhynchites* sp. as detailed hereunder: larvae were collected by spoon in the city of Culiacan, Sinaloa, Mexico (Fig. 1D). Seven larvae were cold immobilized and inoculated (first abdominal segment) with 3 μL of feces. Three inoculated larvae were evaluated immediately after inoculation (time 0), and the rest at 120 h postinoculation (modified from Rosen and Gubler 1974).

The feces supernatants excreted by the larvae exposed to different volumes of infection (70 and 140 μL) were combined and exposed (210 μL) for 120 h to four groups of 30 larvae of *Ae. aegypti* in containers with 15 mL of dechlorinated water.

The total RNA was extracted from the feces and larvae supernatants (SV Total RNA Isolation System Kit; Promega, USA), and a reverse transcription reaction was performed (GoScript Reverse Transcription System; Promega). A PCR was performed with the cDNA, and consensus primers D1 and D2 for DENV. A second amplification was performed with D1 and specific TS4 for DENV-4 (Lanciotti et al. 1992). The amplification conditions proposed by Apodaca-Medina et al. (2018) were utilized.

Results

An amplified a fragment of ~390 bp corresponding to DENV-4 was observed in 50% (2: 4 tests) of the supernatants

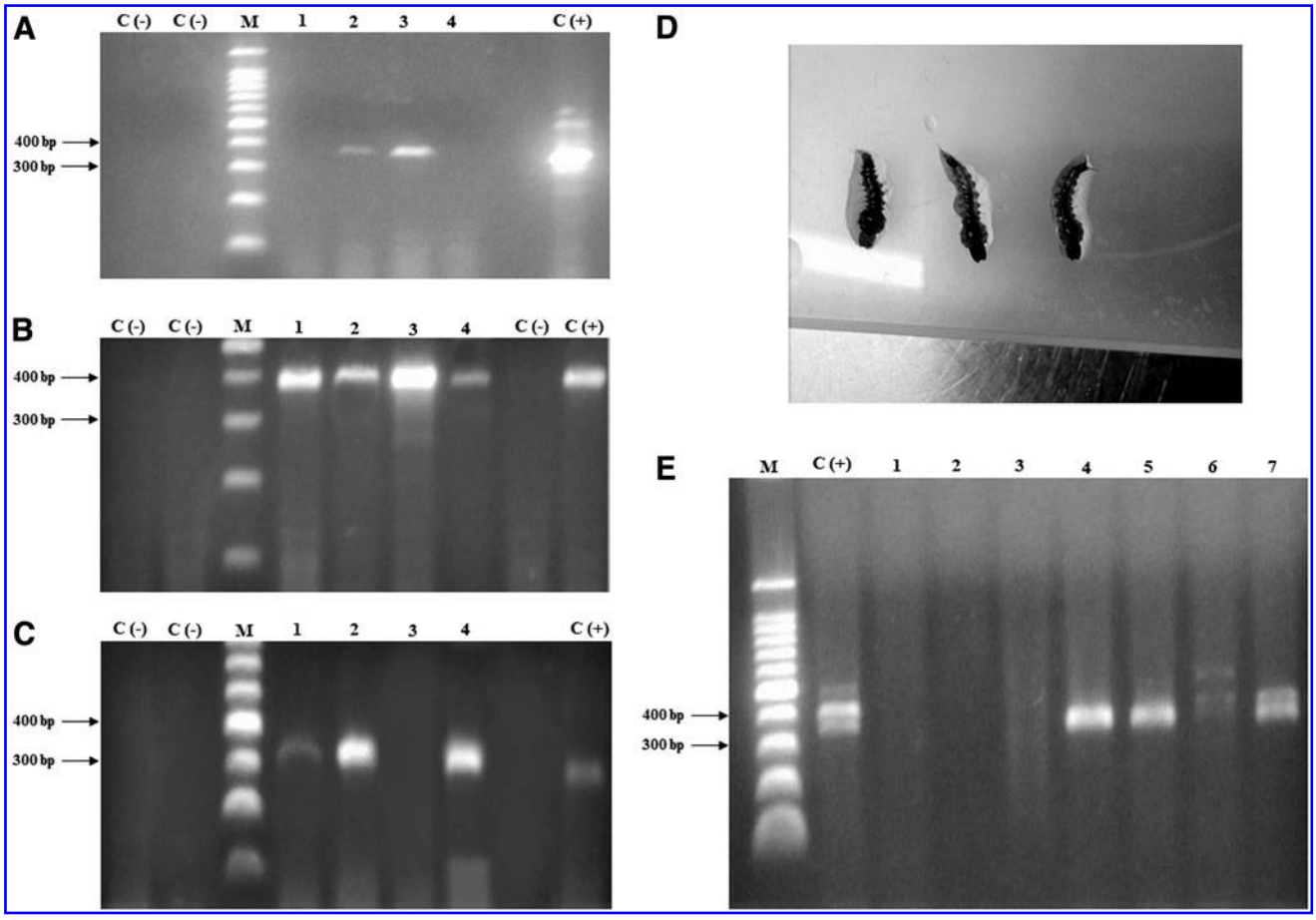

FIG. 1. RT-PCR amplification of DENV-4 in fecal excretions and larvae of *Aedes aegypti*. **(A)** The results of amplification of DENV-4 in feces of larvae exposed to 70 μL are shown; 1–4: samples of feces. **(B)** Results of amplification of DENV-4 in feces of larvae exposed to 140 μL of DENV-4 infected C6/36 cell supernatant; line 1–4 samples of feces. **(C)** Larvae exposed to combined volumes 70 y 140 μL of feces from infected larvae; 1–4 samples of larvae exposed. **(D)** *Toxorhynchites* sp. larvae. **(E)** RT-PCR results of samples of *Toxorhynchites* sp. larvae inoculated with combined feces with DENV-4 of larvae exposed to volumes 70 y 140 μL; line 1–3: 0 h postinoculation, line 4–7: 120 h postinoculation, C (–): negative control of feces and larvae, M: marker 100 bp, C (+): DENV-4. DENV, dengue virus.

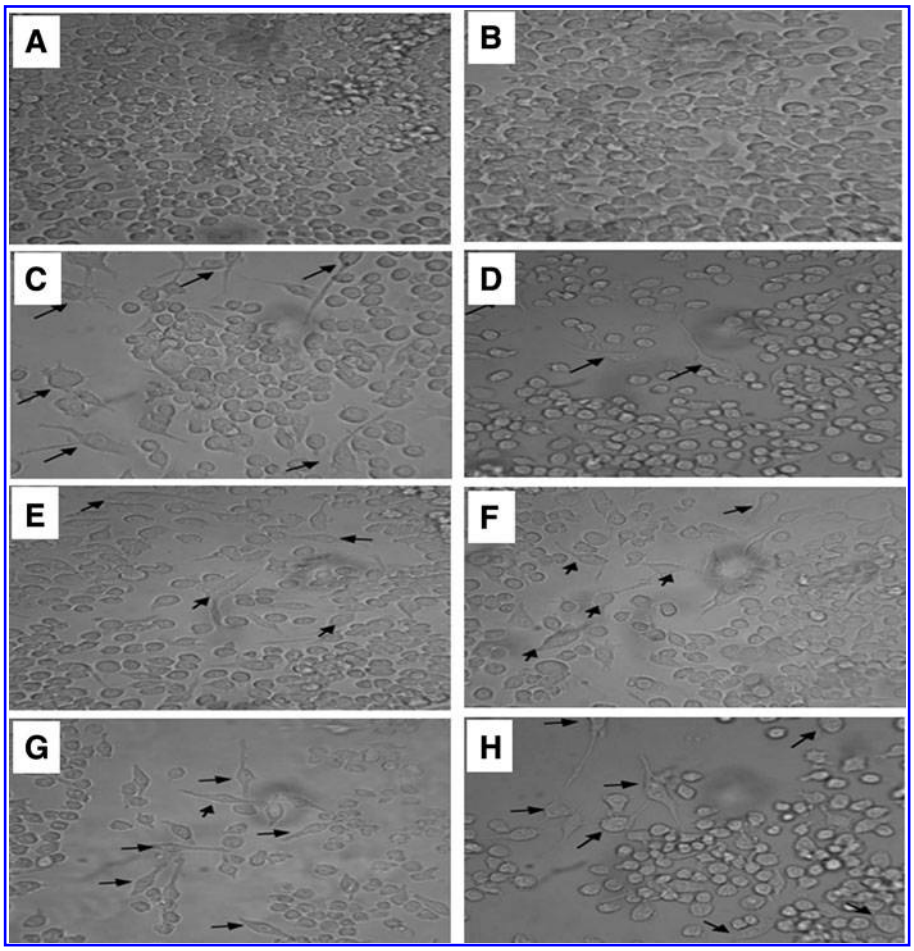

FIG. 2. Effect of infection in RAW 264.7 macrophage at 48 h postinoculation with supernatants from *Ae. aegypti* previously exposed to DENV-4. **(A)** RAW 264.7 cells in DEM. **(B)** RAW 264.7 cells with feces supernatant (negative control). **(C, D)** RAW 264.7 cells exposed to infected C6/36 cell supernatant (positive control). **(E, F)** RAW 264.7 cells exposed to supernatants of feces from larvae exposed to 70 μL. **(G, H)** RAW 264.7 cells exposed to supernatants of feces from larvae exposed to 140 μL. *Arrows* indicate typical characteristics of infected macrophages (magnification 80×).

of feces of larvae exposed to 70 μL (supernatant of C6/36 infected with DENV-4) (Fig. 1A). When the volume to which the larvae were exposed increased to 140 μL, the positivity was 100% (4: 4 tests) (Fig. 1B), indicating a prospective positivity increase dependent on the volume of supernatant exposed to immature *Ae. aegypti*. When these supernatants were inoculated in RAW 265.7 cells, a cytopathic effect was evidenced 24 h postinoculation, indicating infective viability of the virions excreted by the larvae (Fig. 2). Furthermore, the infective viability of the virions was corroborated with the inoculation tests in *Toxorhynchites* sp., where positivity to DENV-4 was observed at 120 h postinoculation (Fig. 1E).

Once the viability of DENV-4 was confirmed in feces supernatants, viral infection tests were carried out in larvae of *Ae. Aegypti*, where was possible to evidence that 75% of the groups exposed to the supernatants were positive for DENV-4 (Fig. 1C).

Discussion

It has been shown that adult *Ae. aegypti* infected with DENV excrete DENV RNA in feces under controlled conditions (Fontaine et al. 2016). This observation, together with the results obtained in this study, support the hypothesis that fecal excretion of DENV RNA is a generalized phenomenon in larvae and adults of *Ae. aegypti* larvae. Likewise, scientific evidence supports the excretion in feces of some arboviruses such as the Ross River virus (RRV) and the West Nile virus (WNV) in adult mosquitoes under laboratory conditions, and in the natural environment (Ramírez et al. 2018, Meyer et al. 2019).

Regarding the viability of infection, Ramírez et al. (2018) reported that RRV and WNV excreted by *Ae. vigilax* and *Culex annulirostrus* have low infectivity rates. In contrast, Aragao and Costa-Lima (1929) argue that the yellow fever virus could be present in feces of *Ae. aegypti* and be viable in monkeys.

The fluctuation of positivity (DENV-4) corresponding to the inoculation assays in *Toxorhynchites* spp. is an indicator of viral replication, and consequently of viability of the viruses exposed to the infection assays. In this case, we determined that the amount of viral RNA in the inoculated larvae (0 h) was insufficient to be amplified by PCR. In comparison, amplification was evident in all cases when processed at 120 h postinoculation, which is evidence of virus replication. Likewise, the cytopathic effect observed in RAW 264.7 cells after 24 h confirms the viability of DENV-4 infection in feces of *Ae. aegypti*.

Our observations indicate that DENV-4 excreted in feces has the capacity to infect *Ae. aegypti* larvae, at least under laboratory conditions. From the point of view of virus-larvae infectious susceptibility, Bara et al. (2013) reported that larval *Ae. aegypti* and *Ae. albopictus* are susceptible to infection when directly exposed to DENV or infected cells.

It has recently been shown that *Ae. aegypti* emerged from immature stages exposed to the Zika virus have the ability

to transmit the virus and infect mice (Du et al. 2019), raising the possibility that this phenomenon occurs under horizontal larvae–larvae transmission induced by viral particles (DENV-4) excreted in feces. In addition, the presence of wild *Ae. aegypti* larvae infected by VT with DENV could influence larvae–larvae transmission in the natural environment and its persistence. However, studies are needed to elucidate the relationship of viral excretion in wild larvae and its public health implications.

Conclusion

The study shows evidence of DENV-4 excretion and transmission in *Ae. aegypti* larvae under laboratory conditions, suggesting a mechanism of horizontal transmission of DENV-4 in larvae.

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Author Disclosure Statement

No conflicting financial interests exist.

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