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Evidence of Vertical Transmission of Dengue Virus in Two Endemic Localities in the State of Oaxaca, Mexico

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Key Words
Transovarial transmission · Dengue · *Aedes aegypti* · Reverse transcription-polymerase chain reaction · Dengue virus

Abstract
Background: Dengue virus is spread in tropical areas of the world and is the causative agent of dengue fever and dengue hemorrhagic fever. It is horizontally transmitted to humans by infected *Aedes* mosquitoes, but it is also able to be vertically or transovarially transmitted to insect progeny. ***Objective:*** In this work, we analyzed the vertical transmission of dengue virus in *Aedes aegypti* mosquitoes collected in two endemic localities in the state of Oaxaca, Mexico. ***Methods:*** The collected larvae were grown in the laboratory and transovarial transmission of dengue virus, either in larvae or newly emerged mosquitoes, was investigated using a semi-nested reverse transcription-polymerase chain reaction method. ***Results:*** Although the presence of dengue virus in larvae could not be demonstrated, the viral genome was amplified in 4 out of 43 pools of in-cage born mosquitoes: DEN 2, 3 and 4 serotypes were detected in 2 pools from Tuxtepec and two from Juchitán. ***Conclusion:*** The results presented here strongly suggest that dengue virus can be vertically transmitted in mosquitoes from Oaxaca, but more studies will be necessary to analyze the epidemiological impact of this mechanism of transmission.

Dengue is the most important mosquito-borne viral disease in the world [1] with 100 million cases of dengue infection, 500,000 cases of dengue hemorrhagic fever (DHF) and 21,000 dengue-related deaths reported per year [1, 2]. This virus is spread throughout the world in tropical areas located between latitudes 30° N and 40° S [2]. The disease displays several manifestations ranging from asymptomatic infection to an influenza-like disease called dengue fever (DF), to a DHF. Some cases of DHF can evolve to dengue shock syndrome which may be fatal [3]. The severity of the disease and the magnitude of the outbreak depend on several factors such as the vector, virus, environment, immunological status of the infected people, and socioeconomic level of the population [4].

Dengue is caused by four serotypes of the single-stranded RNA dengue virus (DEN 1–4), which are members of the *Flaviviridae* family. They are transmitted to

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Table 1. Climate characteristics and monthly collection of *A. aegypti* larvae in Tuxtepec

Month	Rainfall cm^3	Temperature, °	Larvae collection
Jan	7.0	22	–
Feb	13.3	22	–
Mar	2.8	24	–
Apr	0.6	25	450
May	50.8	28	450
Jun	112.4	28	580
Jul	5.7	27	–
Aug	223.8	27	650[1]
Sep	101.5	27	480[1]
Oct	101.8	28	410
Nov	9.0	23	–
Dec	1.0	22	–

Data obtained from www.wunderground.com Public Health Laboratory of Oaxaca.
[1] Pools detected as positive for DEN virus.

Table 2. Climate characteristics and monthly collection of *A. aegypti* larvae in Juchitán

Month	Rainfall cm^3	Temperature, °	Larvae collection
Jan	–	25	–
Feb	–	25	–
Mar	–	26	–
Apr	0.06	28	260
May	–	30	410
Jun	0.08	29	380
Jul	30.6	28	–
Aug	61.5	28	720
Sep	30.7	29	430
Oct	66.9	27	480[1]
Nov	–	26	–
Dec	20.4	25	–

Data obtained from www.wunderground.com Public Health Laboratory of Oaxaca.
[1] Pools detected as positive for DEN virus.

humans by mosquitoes of the *Aedes (Stegomyia)* genera, with *A. aegypti*, *A. albopictus*, *A. polynesiensis* and *A. scutellaris* being the most important [2, 5]. There is not a specific antiviral treatment or vaccine against DEN virus. For this reason, the most efficient method to avoid viral infection is to control its invertebrate vector [4].

The mosquito plays an important role not only in DEN virus transmission, but also as a biological agent that allows for selection of some genotypes over others [6]. Moreover, *A. aegypti* mosquitoes from different geographic origins display different susceptibilities to dengue virus infections [7]. The best documented mechanism of dengue virus transmission is horizontal transmission (human-mosquito); however, vertical or transovarial transmission, where the female-infected mosquito is able to transmit the virus to its progeny, has been reported to occur experimentally [8, 9] as well as in nature [10–14]. Although transovarial transmission has not been routinely determined in wild isolates, likely because of the poor sensitivity of the detection methods [15], the presence of DEN has been demonstrated in nearly 40% of larvae and progeny from *A. albopictus* females that had been orally infected with DEN 2 [16] as well as in *A. aegypti* that had been intrathoracically inoculated with DEN 3 [15]. This mechanism of infection is affected by several factors such as the serotype and strain of the virus, the mosquito species [8] and the line of *A. aegypti* (with high or low susceptibility to DEN) [17].

In 2005, Mexico registered 17,487 cases of DF, 4,418 cases of DHF and 44 related fatalities. 1,200 cases were reported in Oaxaca in 2005: 1,019 of DF and 181 of DHF, which were mainly caused by DEN 1 showing an incidence from 4.81 to 6.39 cases per 100,000 habitants [18].

Since the prevalence of DEN virus in humans and mosquitoes in Oaxaca is one of the highest in Mexico, the participation of transovarial transmission of DEN in *A. aegypti* was evaluated. For our study, we selected two localities, Juchitán and Tuxtepec, where this virus is endemic. During 2005, the period of the study, 493 cases of dengue disease in Juchitán and 248 in Tuxtepec were documented (information kindly provided by the Public Health Laboratory of State of Oaxaca-LESPO). *A. aegypti* larvae were collected from natural sources of human hosts in these two localities. Monthly, a variable number of houses which had been documented for cases of DF or DHF by the National Health Department (Secretaría de Salud) were visited for collection. The larvae from each location were pooled and kept as separate samples. The larvae were mainly collected during two periods: April–June and August–October. A total of 3,020 larvae from Tuxtepec and 2,680 from Juchitán were collected. In Tuxtepec, the highest number of larvae was obtained during June and August which was coincident with the rainfall peak (table 1). Juchitán was a drier location than Tuxtepec and the highest number of larvae was collected in August, one of the months with the highest rainfall index (table 2).

The temperature ranged between 22 and 28° in Tuxtepec (table 1) and between 25 and 30° in Juchitán (table 2). Furthermore, the highest collection of larvae was coincident with a temperature range between 28 and 27° in Tuxtepec and with 28° in Juchitán (tables 1 and 2, respectively).

The presence of dengue virus in the field-collected larvae was evidenced by the Reverse transcription-polymerase chain reaction (RT-PCR) technique which has been used before to detect DEN virus in the saliva of *A. albopictus* females 14 days after an infectious blood meal [16] and in serum samples where attempts to re-isolate the virus by conventional methods had failed [19] supporting its sensitivity to detect DEN [20]. To perform the RT-PCR reported by Seah et al. [19], pools of 20 larvae were placed in a homogenizer (Eppendorf) containing 400 μl of TRIZOL® (Invitrogen) and squashed for 3 min for nucleic acid extraction. After the addition of 100 μl chloroform, the suspension was vortexed briefly and incubated for 5 min at room temperature. The tube was then centrifuged at 8,870 *g* for 5 min at 4° in a microcentrifuge (Eppendorf model 5417R) and the upper phase was recovered and transferred to a new vial containing 250 μl of 2-propanol (J.T. Baker). The mixture was incubated for 5 min at room temperature and kept at –70° overnight. Samples were centrifuged at 12,000 *g* for 10 min at 4°, the pellets were washed with 75% ethanol (Merck), resuspended in 26 μl of RNase-free water, and 10 U of RNase-free DNase I (Roche 776785) were added. After 15 min of incubation at room temperature, the enzyme was inactivated for 20 min at 80°. Finally, the samples were precipitated with ethanol and 3 M sodium acetate, pH 7 (Sigma), and stored at –70°. The integrity of the RNA was evaluated using primers for *A. aegypti* actin mRNA: 5′-ACGTGAAATCGTTCGTGACATTAAG (sense) and 5′-TTAACTTAGAAGCACTTGCGGTGAA (anti-sense). The RT-PCR was performed using the RT-PCR Access® kit (Promega) and a thermocycler (Geneamp PCR System 2400 from PerkinElmer). The RT was performed at 48° for 45 min. Then 35 PCR cycles of 94° for 30 s, 55° for 60 s and 68° for 60 s, and a final cycle of 68° for 10 min were performed (fig. 1b). To detect DEN virus in the samples, primers for the NS3 gene [19] were then used. Briefly, 1 μM of both, the sense DV1 and antisense DV3 DEN primers, 0.2 mM of each dNTP, 1 U of both AMV reverse transcriptase and Tfl polymerase, and 15 ng of total purified larvae RNA were used. The RT was done at 48° for 45 min. Then, 30 PCR cycles of 94° for 30 s, 55° for 60 s and 68° for 60 s were performed. Finally, an additional extension step lasting 10 min at 68° was carried out. To identify the DEN virus serotype in the positively-detected pools, 1 ng of a serotype-specific antisense primer (DSP1–4) previously reported by Seah et al. [19] and 1 ng of the sense primer DV1 were used. The PCR was performed as previously described using the following conditions: one step for 5 min at 94° followed by 30 PCR cycles consisting of 94° for 30 s, 55° for 60 s and 68° for 60 s. All of the samples were analyzed by agarose gel electrophoresis, followed by staining with ethidium bromide and in a UV transilluminator (Cole-Palmer 95500). A negative control, using purified RNA from uninfected C6/36 cells, was included in all reactions (data not shown). To avoid sample contamination some precautions were taken: the nucleic acid extractions and the RT-PCR reactions were performed in different laboratories and the standard dengue strains and the experimental samples were manipulated with a different set of micropipettes.

Initially, we standardized the procedure using different standard DEN virus strains propagated in the laboratory either by intracerebral inoculation in newborn mice

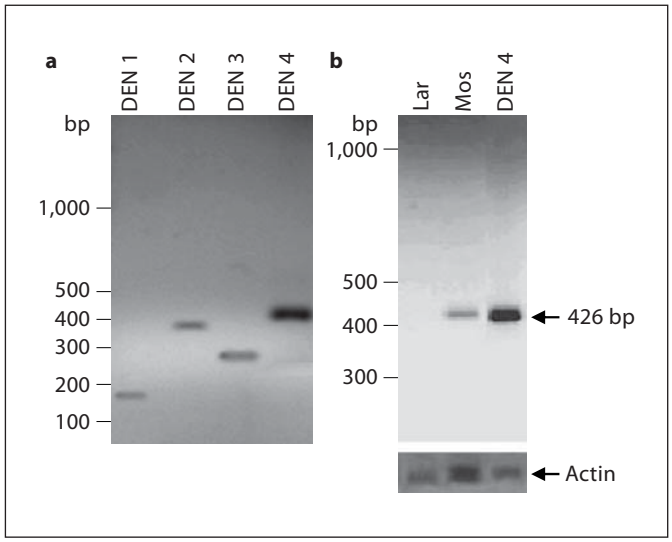

Fig. 1. RT-PCR to detect dengue virus. **a** Agarose gel electrophoresis showing RT-PCR products of NS3 of the different serotypes of dengue virus with the primers DV1 and the type-specific primers DSP1–4. The dengue serotype is indicated in each line. The generated products of 169, 362, 265 and 426 bp specific for dengue virus serotypes 1–4 respectively can be visualized. **b** Agarose gel electrophoresis showing an example of RT-PCR products using total RNA from field-collected larvae and laboratory-cultivated *A. aegypti* mosquitoes. Lar = larvae; Mos = adult mosquito; DEN 4 = dengue virus 4 used as a positive control. In the panel below the *A. aegypti* actin mRNA 518-bp fragment is shown. The molecular markers (λ-ladder 100) are indicated on the left side of both gels.

Table 3. Detection of dengue virus in mosquitoes and larvae of *A. aegypti* collected in Tuxtepec and Juchitán during the year 2005

Stage/location	Larvae[1, 3]		Adult[2, 3]	
	Juchitán	Tuxtepec	Juchitán	Tuxtepec
Total number of specimens examined	280	340	400	460
Positive pools/examined pools	0/14	0/17	2/20	2/23
MIR	0	0	5	4.3
MIR, %	0	0	0.5	0.43
Serotype detected	ND	ND	DEN 4	DEN 2, 3

MIR = Minimum infection rate: (number of positive pools/total specimens tested) × 1,000 [26, 27].
ND = Not determined.
[1] Larvae were collected in several houses of both locations.
[2] Adult mosquitoes were cultured in the laboratory from field-collected larvae and all tested were females.
[3] The total RNA was purified and used in a RT-PCR reaction as described in Methods.

[21] or by infection of C6/36 mosquito cells [22]. The strains used were DEN 1 (Hawaii), DEN 2 (strain donated by Instituto Nacional de Diagnóstico y Referencia Epidemiológicos, Mexico), DEN 3 (H-87) and DEN 4 (H-241). With this technique we were able to amplify a segment of 169 bp for DEN 1, 362 bp for DEN 2, 265 bp for DEN 3 and 426 bp for DEN 4 as was previously reported [19] (fig. 1a).

Once the RT-PCR assay was established, the RNA from 31 pools of 20 specimens each (a total of 620 larvae from both locations) of field-caught larvae were analyzed. Even though the band amplified from actin mRNA was clearly detected in all samples (fig. 1b), suggesting that the method used to isolate RNA was working appropriately, and despite the sensitivity of the RT-PCR method, we could not detect the virus in any of the pools with the conditions used (fig. 1b, lane 1, and table 3). With regard to this, Chow et al. [20] could not detect DEN virus using RT-PCR with larvae of *A. aegypti* that were collected in the field and Rosen et al. [8] could only detect the virus in intrathoracically inoculated *Toxorhynchites amboinensis* mosquitoes 14–19 days after emergence by the head-squash technique. Moreover, other reports show that vertical transmission increases in desiccated eggs from experimentally-infected mosquitoes after 2 months [17]. All of these findings support the conception that the life stage of the mosquito has an important influence on DEN virus infection and that virus replication seems be more efficient in adult mosquitoes than in other stages.

To test the effect of the life stage of the vector in DEN virus detection, the remaining field-collected *A. aegypti* were kept in the laboratory under controlled conditions. The larvae were washed and transferred to 500 ml water containers and fed with fish fodder (aquarium). The water was changed every day to avoid contamination. They were kept in separate cages to ensure that the newly emerged mosquitoes would not have contact with the environment. The newly emerged mosquitoes were alimented with a 5% sugar solution and 10–14 days after emergence, the females were collected and incubated at –20° for 5 min before removal of their wings and legs. The rest of their bodies were used for nucleic acid extraction for RT-PCR reactions as described above. A total of 860 mosquitoes, grouped in 43 pools of 20 individuals each, were analyzed under the same conditions used for larvae and we were able to detect the presence of DEN virus in the adult mosquitoes obtained from collected larvae (fig. 1b, lane 2). These results support the notion that the life stage of the mosquito is an important factor in the detection of DEN virus. Several factors could be responsible for those differences such as the S-phase of the cell cycle in mosquito cells [23], cellular proteins involved in DEN virus replication [24] and the conditions of larvae growth [7] among others; however additional studies will be necessary to test those hypotheses and to develop a more sensitive method to detect DEN virus in larvae and other life-cycle stages of *A. aegypti*.

From all pools that were tested, 4 (9.3%) were positive for dengue virus. Of these, 2 were from Tuxtepec (8.7%), 1 collected in August and the other in September (table 1), and 2 were from Juchitán (10%), from larvae collected in October. In both locations the collection of positive pools was coincident with the rainfall peak (tables 1, 2). The minimum infection rate (MIR) was evaluated as reported

before considering that our collection was made in an essentially infinite population [25–27] with a MIR of 5 for Juchitán and 4.3 for Tuxtepec (table 3). Several studies have proven that transovarial transmission of DEN virus occurs in nature; however, the evidence indicates that it occurs at a very low rate [28]. For example, Khin and Than [10] have reported 5 positive pools out of 283 tested in Rangoon; Fouque et al. reported 3 positive out of 671 in French Guiana [13] and Hull et al. [11] reported 1 positive DEN 4 from 143 pools of eggs collected in Trinidad. In our study, a total of 4 out of 43 pools (20 specimens each) were positive. If we consider that the MIR assumes that a positive pool contains only a single infected insect [27], our results show a slightly higher infection frequency. This could be due mainly to the method used to detect the virus, since the previous studies used immunofluorescence techniques to evidence the presence of DEN instead of the RT-PCR that we used.

Interestingly, DEN 4 was the only serotype detected in Juchitán while DEN 2 and 3 were detected in specimens collected in Tuxtepec (table 3). During 2005, in the state of Oaxaca, the four serotypes of dengue virus had been documented, including the serotypes DEN 2 and 3. Our results are in agreement with these findings because DEN 2 and 3 were the serotypes detected in Tuxtepec. Curiously, we could not detect DEN 1 in any of the mosquitoes tested, even though its presence has been reported in Oaxaca. One possible explanation of this fact is that we noticed that the primers used to detect the serotype DEN 1 were not as sensitive as those used to detect DEN 2–4 (data not shown). On the other hand, the transovarial transmission of dengue virus has been reported to depend on the species of *Aedes* mosquito that was infected and it seems to be less efficient in *A. aegypti* than in *A. albopictus*, especially for DEN 1 [8].

Several findings suggest that even vertical transmission occurs at a very low rate in almost all arboviruses. This is important for both their survival in nature and the establishment of biological transmission [28]. Moreover, it may be an important factor for virus prevalence and maintenance of the virus in nature [15]. Fouque et al. [13] have found *A. aegypti* larvae in plants that were located 30 km from the nearest human settlement in French Guiana supporting the idea that they were infected vertically and that they could be a reservoir for DEN virus. Furthermore, it has been reported that in regions with a seasonal dry period of 3–6 month, like Juchitán in our study, the desiccation-resistant mosquito eggs could be a reservoir for some arboviruses [28].

DEN virus infects non-human primates in a sylvatic environment and humans in urban areas [28]. Phylogenetic analyses [29] have shown that the sylvatic serotypes are ancestors of the endemic/epidemic ones [30] and that they apparently produce neither higher viremia in a mouse model nor a higher level of virus replication in dendritic cells than do the sylvatic strains suggesting that the latter do not require adaptation to replicate more efficiently in the human host [30]. Furthermore, a switch in habitats between the two main vectors of DEN virus, where *A. aegypti* has become more exophilic and less anthropophilic than *A. albopictus,* has been observed in Asia [31]. Both results suggest the possibility that an interchange between the sylvatic and urban cycles could occur and these results indirectly suggest that vertical transmission of DEN virus could have an important role in its epidemiology; however, more epidemiological studies and more sensitive methods to detect this virus are required to confirm the relevance of transovarial transmission in the disease caused by DEN virus.

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