Experimental infection and vector competence of Zika virus in *Aedes aegypti* mosquitoes from Acapulco, Guerrero, Mexico

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Abstract

Objective. To gain a better understanding of the Zika virus (ZIKV) vector transmission in Mexico, we determined the vector competence of a local population of *Ae. aegypti* (Acapulco, Guerrero) for a strain of ZIKV isolated from a Mexican febrile patient. **Materials and methods.** Eggs were hatched and larvae were reared under controlled conditions. After five days post-emergence, female mosquitoes were fed an infectious blood-meal containing ZIKV. Mosquitoes were analyzed at 4, 7 and 14-day post-infection (dpi). Infection (gut), dissemination (wings, legs and heads) and potential transmission (salivary glands) were assessed by RT-qPCR. The Rockefeller *Ae. aegypti* strain was used as ZIKV infection control. **Results.** ZIKV infection, dissemination, and potential transmission rates were 96.2, 96.1 and 93.2%, respectively. **Conclusions.** *Ae. aegypti* (F1) from Acapulco were very susceptible to ZIKV infection, and showed similar vector competence to that of the susceptible Rockefeller strain. To our knowledge, this is the first report of vector competence for ZIKV performed in a Mexican laboratory.

Keywords: vector competence; Zika virus; *Aedes aegypti*; Mexico

Resumen

Objetivo. Determinar la competencia vectorial de una población local de *Ae. aegypti* para transmitir el virus Zika (ZIKV) aislado de un paciente febril mexicano. **Material y métodos.** Se desarrolló la primera generación (F1) de mosquitos *Ae. aegypti* en el insectario a partir de huevos colectados mediante ovitrampas en la Colonia Renacimiento, Acapulco, Guerrero. Después de cinco días de la emergencia, los mosquitos hembras fueron alimentados con sangre infecciosa con ZIKV. La infección (intestino), la diseminación (alas, piernas y cabezas) y la transmisión potencial (glándulas salivales) se evaluaron mediante RT-qPCR, a los 4, 7 y 14 días después de la alimentación. **Resultados.** La infección por ZIKV, la diseminación y las tasas potenciales de transmisión fueron de 96.2, 96.1 y 93.2%, respectivamente. **Conclusiones.** Los mosquitos *Ae. aegypti* (F1) de Acapulco presentan una alta competencia vectorial (93.2%). Según los autores de este estudio, este es el primer informe de competencia vectorial para ZIKV realizado en un laboratorio mexicano.

Palabras clave: competencia vectorial; virus Zika; *Aedes aegypti*; México

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In recent years, viruses transmitted by the *Aedes* mosquitoes like dengue virus (DENV), chikungunya virus (CHIKV), and Zika virus (ZIKV), have spread worldwide causing outbreaks, epidemics and pandemics. These mosquitoes vectors are highly competent for transmission of many arboviruses, although, the molecular factors involved in the vector competence remain elusive.

Zika virus is a single-stranded RNA virus of genus *Flavivirus* and family *Flaviviridae*. In 1947, the virus was isolated from a sentinel rhesus monkey from the Zika Forest in Uganda.[1] At that moment, the virus was circulating among African and Asian populations and it was not considered a public health threat. However, an outbreak in Micronesia in 2007[2] and a major epidemic in French Polynesia and New Caledonia in 2013-2014 alerted health authorities.[3] By 2015, ZIKV infection was reported in Brazil.[4] In 2016, the World Health Organization (WHO) declared a public health emergency regarding the ZIKV infection in Latin America.[5] Since ZIKV discovery, three different lineages have been reported: African, Asian and American.[6] Human infection with ZIKV is mild or asymptomatic in approximately 80% of individuals; nevertheless, the infection has been associated with severe congenital malformations and neurological complications.[7]

ZIKV is transmitted to humans primarily through the bite of infected *Aedes* mosquitoes. However, transplacental, sexual and blood transfusion transmission has also been demonstrated.[8] *Ae. aegypti* Linn. (Diptera: *Culicidae*) mosquitoes are highly competent for ZIKV, therefore, it has been considered as the primary vector in urban environments.[9] The virus has also been detected or recovered from different mosquitoes of genus *Aedes* and *Culex*.[10] However, the role of these species in ZIKV transmission remains controversial.

The susceptibility of mosquitoes to ZIKV infection depends on environmental or abiotic conditions, referred to as extrinsic factors, and the ability of the mosquito to become infected, referred as intrinsic factors. The vectorial capacity of mosquitoes is affected for both sets of extrinsic and intrinsic factors.[11] Vector competence is mainly affected by intrinsic factors, largely depending on the ability of the virus to infect, replicate and disseminate from the mosquito midgut to the salivary glands.[12] The vector competence of *Ae. aegypti* for the transmission of ZIKV has shown great variability. Recently, *Ae. aegypti* populations have been reported to be either refractory or highly susceptible to ZIKV infections.[13]

In Mexico, the first autochthonous cases in humans, and the presence of ZIKV infected mosquitoes were reported in 2015.[14] By 2019, 12 824 confirmed cases of Zika have been reported in the country.[15] Guerrero has been one of the most affected states by ZIKV infection. From 2015 to 2019 a total of 887 individual cases, five Congenital Syndrome cases,[16] and six Guillain-Barre syndrome cases have been confirmed in the State.[17] To assess the current and future public health risk imposed by arboviruses in Mexico, in this pilot study, we determined the vector competence of a local population of *Ae. aegypti* from Acapulco for a strain of ZIKV isolated from a febrile patient from Mexico. To evaluate the vector competence, the infection, dissemination and transmission rates were estimated. To our knowledge, this is the first report of vector competence to ZIKV performed in a Mexican research facility and a circulating ZIKV from the same country.

Materials and methods

Ethics statement

This study was approved by the Ethics Review Committee of National Institute of Public Health (*Instituto Nacional de Salud Pública*, INSP) under the protocol number 909 CI. The experimental work was conducted from August 2017 to July 2018 at the insectary of INSP in Cuernavaca, Morelos, Mexico.

Mosquito collection

We carried out the collection of *Ae. aegypti* eggs at Acapulco (Colonia Renacimiento), Guerrero, Mexico, in collaboration with the National Center of Preventive Programs and Disease Control (Cenaprece, in Spanish). Ovitrap surveys are a common practice in this locality, with a 10-15% of city blocks coverage. It is performed according to the routine policies of the Mexican Vector Control Program (NOM-032-SSA2–2014). The clutches of eggs attached to the oviposition substrate paper were transported, maintained and grown in insectary conditions at INSP. The Rockefeller strain of *Ae. aegypti* was obtained from the INSP mosquito collections, and used as internal control in all experiments.

Eggs were hatched and larvae were reared at a density of 200 larvae in 2-liter plastic trays. Larvae were fed with a rat chow, yeast extract and lactoalbumin hydrolysate liquid diet (1:1:1 mix; 25g/200ml). Adults were maintained in rearing in plastic cages and fed on cotton soaked with 10% sucrose solution. The rearing conditions were 30 ± 1°C and 50-60% RH with a 12:12 LD. Adult mosquitoes five days old were obtained for the experiments. The first generation (F1) of mosquitoes obtained in the laboratory was used in the experiments.

Virus

The virus used in the experiments was isolated in our laboratory from a serum of a Mexican patient (from Yucatán) with confirmed ZIKV infection. The serum was kindly provided by Dr. Esteban Muñoz-Medina from the Epidemiological Surveillance Laboratory at the Mexican Institute for Social Security (*Instituto Mexicano del Seguro Social*, IMSS). The virus was passed once on *Ae. albopictus* C6/36 cells. Briefly, cells were grown at 28 °C in Leibovitz's L-15 medium containing 10% heat inactivated fetal bovine serum (FBS) and 10% tryptose phosphate broth. Cell monolayers in 75 cm² flasks (80% confluence) were infected with a multiplicity of infection (MOI) of 0.1 and incubated for 60 min at 28 °C. Then, the cellular monolayer was supplemented with L-15 medium and incubated at 28 °C for five days. This procedure was repeated three times until cytopathic effect appeared. The identity of the ZIKV was checked by RT-PCR and the amplified fragment was sequenced.

Titration of viral stock

The viral titer was calculated via plaque assay on VERO cells. Cells were seeded in 24-well culture plates and grown at 80% confluence in Dulbecco´s Modified Eagle´s Medium (DMEM) (Sigma), 5% FBS and 1% L-Glutamine. Plates were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for two days. Cells were infected with ZIKV serial dilution in DMEM (10^{-1} to 10^{-8}). After 90 min of virus absorption, the virus inoculum was retired and the cells were supplemented with DMEM, 5% FBS and 1.5% carboxyl methylcellulose (Sigma-Aldrich). Plates were incubated at 37 °C with 5% CO_2 for seven days. Finally, the medium was retired and the cells were fixed with 1% paraformaldehyde for 30 min and stained with 1.5% crystal violet solution (Sigma-Aldrich). The viral titer was expressed as Plaque-Forming Unit per milliliter (PFU/mL).

Infection of mosquitoes

Oral infection was performed in a BSL-2 laboratory, with the ZIKV concentration reported previously.[18,19] 3-5 day-old mosquito females were allowed to feed for 30-60 min through a membrane feeding apparatus. The virus was diluted in rabbit blood (1:1 proportion; with a final viral concentration of 1×10^6 PFU/mL) and maintained at 37 °C by a warm water circulation. Engorged female were separated in groups of 50 mosquitoes and maintained under insectary conditions as mentioned above.

Mosquitoes were individually processed at 4, 7 and 14-days post infection (dpi). At each time, midguts were dissected in order to evaluate viral load and infection rate (IR), estimated as the number of ZIKV-positive midguts with respect to the total number of fed females. Legs, wings and head (LWH) were tested to assess viral load and dissemination rate (DR), calculated as the number of the mosquitoes with ZIKV-positive legs, wings and head among the number of mosquitoes with ZIKV-positive midgut. The salivary glands were dissected to calculate viral load and potential transmission rate (PTR), defined as the number of mosquitoes with ZIKV-positive salivary gland among the number of mosquitoes with ZIKV-positive midguts. Control mosquitoes were fed with a non-infected blood meal. All tissues and body parts were stored in RNA later (Ambion), and preserved at -70 °C.

Zika virus detection

Tissues and body parts were homogenized separately using a Bio-vortexer (Daigger) homogenizer. The RNA extraction was done using the QIAamp Viral RNA mini kit (Qiagen), according to the manufacturer protocol. One-Step RT-qPCR Kit (NEB, Luna Universal; Bio-Labs) was used to prepare all PCR reaction systems. To amplify a region of 100 nucleotides at the viral protein NS4B, the sequences of primers used in PCR reactions were: forward: 5´-CAGCTGGCATCATGAAGAAYC-3´, reverse: 5´-CACYTGTCCCATCTTYTTCTCC-3´, probe: CYGTTGTGGATGGAATAGTGG, as previously described.[20] One step RT-qPCR was performed in a 25 μl reaction mixture containing: 12.5 μL One-StepReverse Transcriptase qPCR Master Mix, 900 nM forward primer, 900 nM reverse primer, 200 nM probe and 5.0 μL of RNA samples plus a required volume of nuclease-free water (Ambion) to adjust the final reaction volume to 25 μl. RT-qPCR was performed under the following conditions: reverse transcription at 55 °C for 10 min, initial denaturation at 95 °C for 1 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing and extension at 60 °C for 30 s. Positive (RNA from cell culture supernatant) and negative (water) controls were also included for both the RT and PCR steps.

Absolute quantification

For standardization of the assay, a plasmid of ZIKV (NS4B region) was used. The concentration was calculated accurately from optical density measurements of plasmid DNA (molecules/μl) as follows: $C \times A/L$, where C represents the concentration of RNA (g/mL) assessed by OD measurement, A is the Avogadro number (6.023×10^{23}), L is the length of the synthetic RNA (nucledotide), and 330 is an approximation of the molecular weight

of a nucleotide (g/mol). Serial dilutions, ranging from 1.88 x 10^9 to 4.81 x 103 copies were made. The results of measurements of each standard concentration were used for the design of a standard curve for mean Ct values in order to calculate copy numbers in samples. Samples with Ct < 32 were considered positive. All qPCR experiments were performed using StepOne Real-Time system from Applied Biosystems.

Statistical analyses

Four sets of analyses were performed. For the first set, Kruskall-Wallis *H* and Mann-Whitney U tests were performed to compare the day post-infection effects in the ZIKV viral load separately for each tissue and mosquito strain. For the second set, Kruskall-Wallis *H* and Mann-Whitney U tests were run to compare viral load at 4, 7 and 14 dpi among tissues (separately for each mosquito strain separately). For the third set, Kruskall-Wallis *H* and Mann-Whitney U tests were used to detect significant differences in viral load between mosquito strains independently for each tissue. For the fourth set, the IR, DR and PTR between strains were tested (at each dpi) by the Pearson's Chi-square (x^2) test. Statistical analyses were performed with Graph Pad Prism v7.02 and Stata v14.

Results

In order to evaluate the susceptibility of the *Ae. aegypti* Acapulco strain to ZIKV infection, we artificially fed mosquitoes with a mix of blood with 1x10^6 copies of ZIKV as described in the methods section. Only the female engorged was used for the dissection of tissues group: midgut; LWH; and salivary glands at 4, 7 and 14 days post infection (dpi) of each mosquito exposed to the ZIKV. We evaluated the percent of infection in the midgut, the percent of dissemination in LWH and the percent of putative transmission in salivary glands according to a previous report.[21] In all the experiments, we used Rockefeller strain as positive control due its susceptibility to ZIKV infection.[22]

For *Ae. aegypti* Rockefeller strain, differences between 4 and 7 dpi and between 7 and 14 dpi for all tissues were detected (figure 1). In all tissues, the maximum viral load was observed at 7 dpi ($p<0.01$). However, it was higher in midguts ($p<0.01$). At 14 dpi we detected a decrease in viral load for all tissues (figure 2).

For the *Ae. aegypti* Acapulco strain, results showed an early ZIKV infection (at day 4 dpi) in all tissues. However, viral load was higher in midguts (1x10^4-1x10^8 copies/μl) than in LWH and salivary glands ($p<0.01$). The maximum viral load was detected at 7 dpi. A sig-
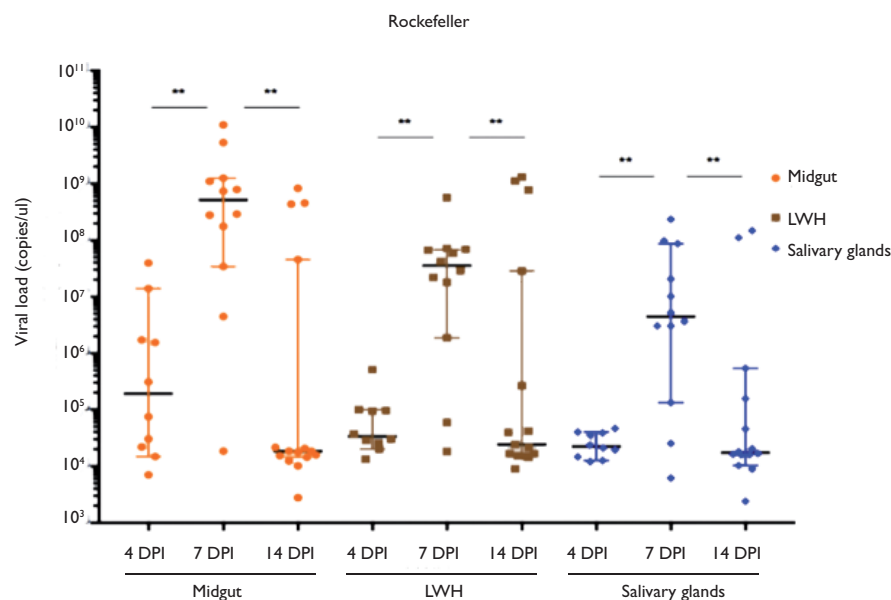
Rockefeller

ZIKV detected in midguts, tissues (LWH: legs, wings and heads) and salivary glands of mosquitoes *Ae. aegypti* Rockefeller strain at 4, 7 and 14 dpi. Each point represents the number of copies of ZIKV by individual mosquitoes. The lines represent the mean with an 95% interval confidence; Mann-Whitney and Kruskall-Wallis test. The Graph represent one of three independent assays.
* $p<0.01$

Figure 1. Number of copies of ZIKV RNA by rt-qPCR in individual mosquito *Ae. Aegypti* Rockefeller strain
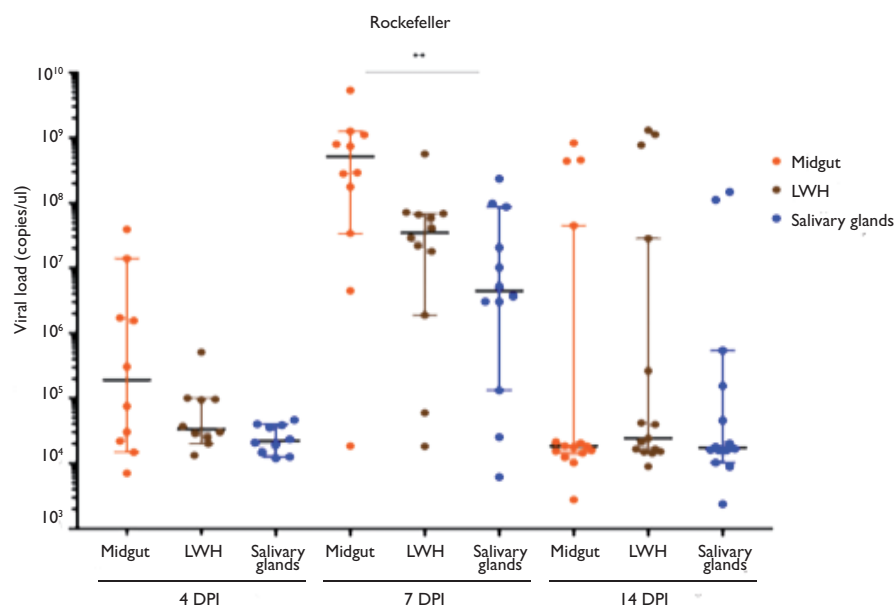
The graph shows the comparison between the ZIKV load to different dpi in each tissue of individual mosquitoes. Each point represent the number of copies of ZIKV by individual mosquitoes. The lines represent the mean with an 95% interval confidence; Mann-Whitney and Kruskall-wallis test. The graph represents one of three independent assays.

* *p*<0.01

LWH: legs, wings and heads

FIGURE 2. INFECTION, DISSEMINATION AND PUTATIVE TRANSMISSION RATES IN MOSQUITOES *AE. AEGYPTI* ROCKEFELLER STRAIN

nificant increase of viral load from 4 to 7 dpi in LWH (*p*<0.01) and salivary glands (*p*<0.05) was detected. At 14 dpi we detected a decrease in viral load for all tissues. Despite a viral load increase in midguts at 7 dpi was observed, no difference among dpi were detected (figure 3). Results indicate a similar dynamic infection between the Rockefeller and Acapulco strains (figure 4).

Vector competence

The Rockefeller strain showed an IR of 92.5%, while the Acapulco strain IR was 96.2%, suggesting a higher susceptibility for the wild strain. PTR comparison indicated that Rockefeller strain had a higher transmission rate (97.2%) than the wild strain (93.2%). However, there is no significant difference between strains (table I). Both strains showed the maximum virus load at 7 dpi, and the same susceptibility to ZIKV infection.

Discussion

In this study, we report that *Ae. aegypti* mosquitoes collected in Acapulco, Guerrero, Mexico were highly competent for a ZIKV strain isolated from a Mexican

patient. Our results showed that the Acapulco strain has similar IR, DR and PTR to the Rockefeller strain (highly susceptible to ZIKV infection).[22] Moreover, we observed a higher IR for the Acapulco strain. At 14 dpi, DR and PTR were lower than those of the Rockefeller strain; however, viral load was higher for the wild strain. The Acapulco strains display high percent of infection at 7 dpi in all tissues, suggesting that the intrinsic incubation period could be shorter than previously reported.[21] Interestingly, our data revealed high IR, DR and TR individual variation, which is similar to the data from previous reports of mosquitoes infected with dengue virus.[23,24] Consequently, we hypothesized that each mosquito in field conditions has a different response to ZIKV infection, similar to the variability observed in humans.

A recent report was published while this manuscript was being prepared, that shows large variance (8-51%) for vector competence for ZIKV in *Ae. aegypti* from different regions of Mexico.[19] In the same study, despite mosquitoes were infected with a non-Mexican viral strain, the authors reported a 90% ZIKV salivary gland infection rate of *Ae. aegypti* from Guerrero at 14 dpi, comparable to data obtained in our work (93.2%).
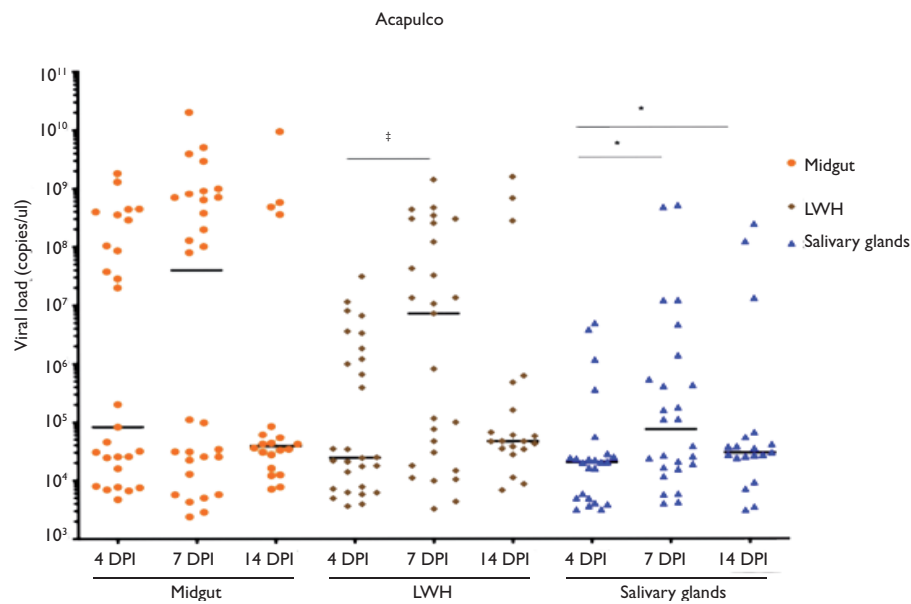
Acapulco

ZIKV detected in midguts, tissues (LWH: legs, wings and heads) and salivary glands of mosquitoes *Ae. aegypti* Acapulco strain at 4, 7 and 14 dpi. Each point represents the number of copies of ZIKV by individual mosquitoes. The lines represent the mean with an 95% interval confidence; Mann-Whitney and Kruskall-wallis test. The graph represents one of three independent assays.
* *p* < 0.05
‡ *p* < 0.01

Figure 3. Number of copies of ZIKV RNA by rt-qPCR in individual *Ae. aegypti* mosquitoes Acapulco strain
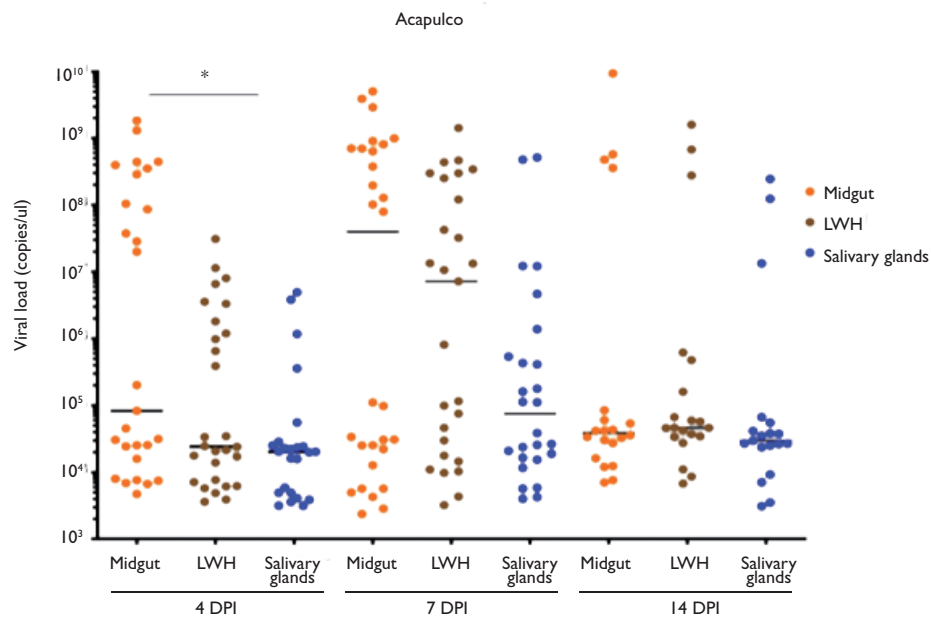
Acapulco

The graph shows the comparison between the ZIKV load to different dpi in each tissue of individual mosquitoes. Each point represent the number of copies of ZIKV by individual mosquitoes. The lines represent the mean with an 95% interval confidence; Mann-Whitney and Kruskall-wallis test. The graph represents one of three independent assays.
* *p* < 0.01.

Figure 4. Infection, dissemination and putative transmission rates in mosquitoes *Ae. aegypti* Acapulco strain

Table I

**Infection, dissemination and potential transmission rate in *Ae. aegypti*
Rockefeller and Acapulco strain**

	Rockefeller			Total	Acapulco			Total
	4 dpi n=10 (%)	7 dpi n=15 (%)	14 dpi n=15 (%)	R n=40 (%)	4 dpi n=30 (%)	7 dpi n=30 (%)	14 dpi n=20 (%)	A n=80 (%)
Infection rate	10/10 (100)	12/15* (80)	15/15 (100)	37/40 (92.5)	27/30 (90)	30/30* (100)	20/20 (100)	77/80 (96.2)
Dissemination rate	10/10 (100)	12/12 (100)	15/15 (100)	37/37 (100)	27/27 (100)	27/30 (90)	20/20 (100)	74/77 (96.1)
Potential tranmission rate	10/10 (100)	12/12 (100)	14/15 (93.3)	36/37 (97.2)	24/27 (88.8)	26/27 (96.2)	19/20 (95)	69/74 (93.2)

The table shows the individual number at different dpi (n) and the percent by X2.
* $p < 0.05$.

Also, a 50% transmission rate (saliva infection) was observed at 14 dpi. In our study, we were unable to collect saliva (usually obtained to quantify transmission rates), but since salivary glands infection were similar and viral load decreased (at 14 dpi), a similar TR in saliva could be expected for our strain.

Considerable work has recently been undertaken to understand *Ae. aegypti* vector competence for ZIKV. Different variables influence vectorial competence of mosquitoes, however genetic and environmental factors of both the vector and the pathogen play decisive roles. Likewise, present knowledge also indicates a possible effect of gut microbiota.[13] Different studies have reported a great variability for vector competence among mosquito populations from different geographic origins (from non-susceptible to 100% susceptible).[19-26] In their review, Souza-Neto and colleagues[13] recommended that standardized procedures must be implemented to obtain comparable and reproducible results. For this reason, in this pilot study, we established the infection procedure in our insectary facility (controlled environmental conditions) with a virus isolated in Mexico, and an optimal viral concentration was used as reported previously.[18]

ZIKV continues expanding in Mexico, although a decrease in the number of Zika disease human cases has been reported. It is probable that herd immunity has been decreasing the number of cases, however, since the emergence of a new naïve human generation, future outbreaks are expected.[27] Because there is great variability in susceptibility to infections across geographic populations, vector competence determination is a key parameter in evaluating the risk of arbovirus transmission and spread. Therefore, the results of our study show two important facts: 1) *Ae. aegypti* from Aca-

pulco, Mexico (an important national and international tourist destination), is a competent vector of ZIKV and is responsible for the Zika endemic cases[15] and 2) new naïve people in this area will be at risk of ZIKV infection and outbreaks. This may have great relevance considering that Acapulco is the first tourist destination for the inhabitants of Mexico City, due to its geographical proximity.

In our country, integrated vector control has included the academic biomedical research community, mainly in the implementation of risk-based protective programs and strategies. This has been enhancing the nation's vector management actions and encouraging the development of projects in Mexican research facilities. We are convinced that success in the control of arboviruses relies on strengthening the relationship between public health, federal and state programs and academic/research institutions. Due to the great variability in susceptibility to arboviral infections across geographic populations of mosquito *Aedes spp.*, in the near future, we hope to have an entomological risk map that includes vectorial competence data for arboviruses dissemination in Mexico.

Funding

Declaration of conflict of interests. The authors declare that they have no conflict of interests.

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