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Research

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Wolbachia in Native Populations of *Aedes albopictus* (Diptera: Culicidae) From Yucatan Peninsula, Mexico

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Abstract

This study reports the results of a molecular screening for *Wolbachia* (*Wb*) infection in *Aedes albopictus* (Skuse) populations recently established in the Yucatan Peninsula, Mexico. To do so, collections of free-flying adults with BG traps and emerged adults from eggs after ovitrap field collections were performed in three suburban localities of the city of Merida, Yucatan. Overall, local populations of *Ae. albopictus* present a natural *Wb* infection rate of ~40% (18 of 45). *Wb* infection was detected in both field-collected adults (76.5%, 13 of 17) and eggs reared (17.8%, 5 of 28) and in 37.9% (11/29) of females and 43.7% (7/16) of male *Ae. albopictus* mosquitoes. An initial screening for *Wolbachia* strain typing showed that native *Ae. albopictus* were naturally coinfected with both *wAlbA* and *wAlbB* strains. The knowledge of the prevalence and diversity of *Wolbachia* strains in local populations of *Aedes* mosquitoes is part of the baseline information required for current and future *Wolbachia*-based vector control approaches to be conducted in Mexico.

Key words: *Wolbachia*, *Aedes albopictus*, *Aedes aegypti*, *w*AlbA, *w*AlbB

Aedes (*Stegomyia*) *albopictus* (Skuse), commonly known as the 'Asian tiger mosquito', is considered a secondary but competent vector of arboviruses of great importance in public health including dengue (DENV), chikungunya (CHIKV; Bonizzoni et al. 2013), and Zika (ZIKV; McKenzie et al. 2019). Although it is native to Asia, it has invaded and colonized many countries in the Americas, Europe, and Africa (Battaglia et al. 2016, Kraemer et al. 2019). In Mexico, *Ae. albopictus* has been recorded in 16 states including Mexico City, Guanajuato, Jalisco, Coahuila, Chiapas, Hidalgo, Morelos, Nuevo León, Oaxaca, San Luis Potosí, Sinaloa, Tabasco, Tamaulipas, Veracruz, Yucatán, and Quintana Roo (Villegas-Trejo et al. 2010, Salomón-Grajales et al. 2012, Ortega-Morales et al. 2018, Contreras-Perera et al. 2019, Dávalos-Becerril et al. 2019, González-Acosta et al. 2020), but its geographical distribution is expected to increase because the macro- and micro ecological conditions suitable for its establishment, particularly throughout South Mexico (Pech-May et al. 2016, Yañez-Arenas et al. 2018).

Wolbachia (Rickettsiaceae) (hereafter called as *Wb*) are ubiquitous obligate bacterial endosymbionts that naturally infect ~20% of all insect species (Hilgenboecker et al. 2008). *Wb* is maternally transmitted in insects, and it is known to exert a profound impact on host biology, mainly at the reproductive level. The most common reproductive effect is called cytoplasmic incompatibility (CI). *Wb*-induced CI modulates the production of viable eggs once an uninfected female mates a *Wb*-infected male, whereas *Wb*-infected females can successfully breed with either infected or uninfected males (Werren et al. 2008).

Wb naturally infects many mosquito species including *Ae. albopictus* but not the primary dengue vector *Aedes aegypti* (Ross et al. 2020). *Wb* introduced into *Ae. aegypti* via transinfection can cause early embryonic arrest (CI) and egg hatch failure (Mains et al. 2019). Interestingly, *Wb* can also cause pathogen interference in *Ae. aegypti* as *Wb*-infected mosquitoes are less susceptible to be infected or coinfected with important arboviruses such as DENV, CHIKV, ZIKV, Mayaro virus, and yellow fever virus (Moreira et al. 2009,

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Walker et al. 2011, Aliota et al. 2016, Pereira et al. 2018). This has led to inundative field-releases of *Wb*-infected mosquitoes as a potential control strategy for *Ae. aegypti*, either by population suppression (Crawford et al. 2020) or by population replacement (World Mosquito Program 2017, Nazni et al. 2019, Ryan et al. 2019).

In Mexico, both *Wolbachia*-based approaches, one using *Wb* strain *wMel* (*Wolbachia pipientis* from *Drosophila melanogaster*; Dutra et al. 2015, Nguyen et al. 2015) for population replacement in Baja California, and another using *w*AlbB (*Wolbachia pipientis* from *Ae. albopictus*; Xi et al. 2005) for population suppression in Yucatan, are under initial phases of implementation and evaluation for the control of *Ae. aegypti*. Data on natural infection frequency are critical to evaluate the potential of using *Wolbachia* as a vehicle to modify insect vector populations (Turelli and Hoffmann 1999, Kitrayapong et al. 2002). However, the prevalence and characteristics of *Wolbachia* in natural mosquito populations are yet poorly known in Mexico.

Aedes albopictus was recently reported in the periphery of Merida, the capital of the state of Yucatan, and the city with the largest population and major epidemiological importance in the Peninsula of Yucatan for the transmission of DENV, CHIKV, and ZIKV (Contreras-Perera et al. 2019). *Aedes albopictus* is very soon expected to invade and coexist with populations of *Ae. aegypti* in Merida. Thus, this study showed the molecular screening for *Wolbachia* infection of field-collected adult *Ae. albopictus* mosquitoes in the suburban areas of Merida, Yucatan.

Materials and Methods

Study Area

Field collections of local *Aedes* populations (adults and eggs) were performed every week from April to December of 2019 at San Pedro Chimay (20°51′55″N 89°34′46″O), Hacienda Tahdzibichen (20°53′06″N 89°35′52″O), and Tekik de Regil (hereafter Tekik; 20°48′59″N 89°33′39″O), all suburban areas in the periphery of the city of Merida in the Peninsula of Yucatan (southeast Mexico; Fig. 1A). Sociodemographic features of these localities include an average of 1,200 inhabitants per locality with an average of 6 households and 31 inhabitants per hectare (INEGI 2016). They share similar urban and ecological landscapes such as type of housing and share large vegetated backyards with vegetation (coverage > 60%). The average altitude of the localities is 9 m above sea level. The climate is mainly warm with an annual average temperature of 26°–27°C (36°C max–18°C min), relative humidity of 70–75%, and two distinct climate phases during the year: a rainy season, from May/June to October with a rainfall of 882.5 mm, and a dry season, from November to April with rainfall of 167.9 mm (INEGI 2017).

Mosquito Collection and Rearing

Aedes adults were collected using outdoor BG-sentinel traps (20–30 traps per locality) for 24 h/periods, one per week), as part of the routine surveillance for control of *Ae. aegypti* in the suburban communities of Merida performed by the Collaborative Unit for Entomological Bioassays (UCBE) and the Universidad Autonoma de Yucatan (UADY). According to the CDC and other studies, BG-sentinel traps are currently the most used and the gold-standard adult traps for the sampling, monitoring, and surveillance of outdoor *Aedes* and *Culex* species in field trials (Li et al. 2016, CDC 2018). Collected specimens were transported to UCBE-UADY for their identification using standard taxonomic keys (Rueda 2004). As part of the vector control program protocol in Mexico, corroboration and validation of larvae and adult specimens of mosquitoes is supported by the National Reference Center at the Instituto de Diagnóstico y Referencia Epidemiológicos (InDRE) of the Ministry of

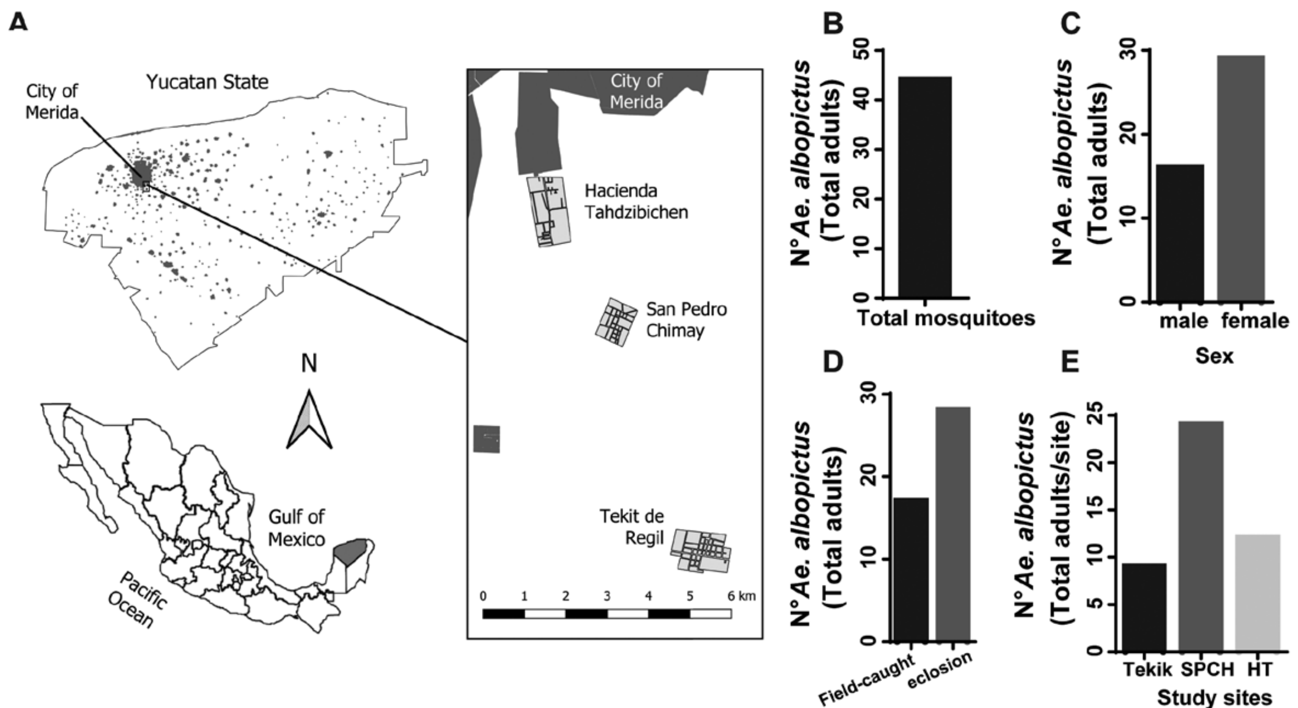

Fig. 1. Study area: distribution and collection sites of *Aedes albopictus* in suburban areas of Merida, Yucatan. (A) Location of localities used for field-collection of *Aedes albopictus* adults and eggs. (B) Total of individual adult mosquitoes included in this study and their distribution by (C) sex, (D) collection method, and (E) locality.

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Health in Mexico. Voucher specimens are deposited at the Colección Entomológica Regional (CER)—Universidad Autónoma de Yucatán (UADY). All *Ae. albopictus* were separated from *Ae. aegypti*, which is also present in the localities. *Aedes* eggs were collected using ovitraps (approximately one ovitrap per Ha) in each locality and served weekly. Paper strips with eggs were sent to the LCB-UADY for mosquito rearing following standard operating procedures of the LCB-UADY. Briefly, eggs were incubated for embryo development (48 h) at room temperature. Larvae were reared and fed 6% larvae feeding solution (Food product for tilapia [Biofinguerlin]: yeast powder [Pronat Ultra], 9:1, respectively) in plastic containers with water. Pupae obtained were maintained within BugDorm-1 Insect Rearing Cages. Adults emerged were maintained for 24 h under standard insectary conditions at 80 ± 5% humidity, 26 ± 1°C, and 12/12 light/dark cycle. Then, specimens were cold immobilized for taxonomic classification and stored at −20°C until further analyses.

DNA Extraction and PCR Screening for *Wolbachia* Infection

Total genomic DNA from individual adult mosquitoes was extracted using a Blood and Tissue DNEasy Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions with some in-house modifications. Briefly, individual adult mosquitoes were firstly disinfected inside sterile Eppendorf tubes (70% ethanol) at room temperature (2 h) and later mechanically homogenized using a sterile pestle and electric homogenizer after adding the lysis buffer from the DNA extraction kit (Qiagen). After elution, DNA was quantified using a nanodrop (Thermo Scientific) and stored at −20°C until further analyses. To detect *Wolbachia* infection in *Aedes* mosquitoes, an initial set of primers was used to specifically amplify the 16S rRNA from *Wolbachia* as previously described: 16-2F (5′-AGC TTCGAGTGAAACCAATTC-3′) and 16-2R (5′-GAAGATAATGAC GGTACTCAC-3′; Simoes et al. 2011). An end point PCR protocol was performed using a Mastercycler EP Gradient-Thermal-Cycler (Eppendorf) and *Taq* DNA polymerase recombinant kit including PCR buffer (10×), $MgCl_2$ (50 mM), dNTPs mix (10 mM), forward and reverse primers (10 µM), *Taq DNA polymerase* (5 U/µl), and extracted DNA template (100–200 ng per reaction). Amplification parameters were established as follows: initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 1 min, Tm annealing at 55°C for 1 min, and extension at 72°C for 1 min; final extension at 72°C for 3 min. Genomic DNA extracted from a local strain of *Ae. aegypti* infected with *Wolbachia* strain B (hereafter referred to *w*MIDB, F9, kindly donated by Dr. Xi, Michigan State University) as previously described (Xi et al. 2005) was used as PCR-positive control. On the other hand, DNA extracted from *Wolbachia*-free (*Wb*) *Ae. aegypti* strain collected from Yucatan (hereafter referred to *wt*MID, F4) was used as the negative control. Amplified DNA was visualized using agarose gel (1.5%) stained with Saber safe (Thermo Scientific) and UV excitation. The amplified 16S rRNA gene was approximately 1,000 bp.

Molecular Typing of *Wolbachia* Infection in *Ae. albopictus*

A set of samples that were positive using the 16-2 primers described above were additionally processed for typification of the *Wolbachia* strain in infected *Ae. albopictus* mosquitoes using two sets of primers previously described to specifically amplify the *Wolbachia surface protein* (*wsp*) gene from strains A and B (Zhou et al. 1998, Ahmad et al. 2017). Briefly, a PCR mixture was prepared to contain extracted DNA template (200 ng per reaction), PCR buffer (10×), $MgCl_2$ (50 mM), dNTPs mix (10 mM), *Taq DNA polymerase* (5 U/µl), RNAse/DNAse-free water, and forward and reverse primers (10 µM) to amplify DNA genome from *Wolbachia* strain A and B described as follows: primers 328F (5′-CCAGCAGATACTATTGCG-3′) and 691R (5′-AAAAATTAAACGCTACTCCA-3′) for *w*AlbA strain, and primers 183F (5′-AAGGAACCGAAGTTCATG-3′) and 691R (describe above) for *w*AlbB. Amplification parameters were established as described above for 16-2 primers. DNA extracted from *Ae. aegypti* mosquitoes either *Wb* free (*wt*MID) or *Wb* strain B infected (*w*MIDB) was used as negative and positive controls for the PCR assay, respectively, following published protocols (Xi et al. 2005). These two sets of primers amplify a DNA fragment ranging from 400 to 600 bp depending on the individual *Wb* strain.

Results and Discussion

In total, 45 *Ae. albopictus* adult mosquitoes (64% female–36% male), among of which was 17 captured with BG-sentinel traps and 28 reared from field-collected eggs using ovitraps, were examined for *Wb* infection by PCR from the three localities (Fig. 1A–D). The trapping *Ae. albopictus* mosquitoes in both adult and egg stages provide further evidence of the early invasion of this mosquito species in the suburban areas of the municipality of Merida (Salomón-Grajales et al. 2012, Ortega-Morales et al. 2018, Contreras-Perera et al. 2019).

In this study, using a universal set of primer nucleotides to amplify a sequence of the *Wb* 16S rDNA gene (Table 1), we initially assessed the circulation of *Wb* in native *Ae. albopictus* mosquitoes from all the localities, either from field-caught and free-flying adults or those reared from eggs (Fig. 2). This initial PCR assay resulted in the amplification of approximately 1,000 base pairs long DNA fragment, identified as positive for *Wb* infection (Fig. 2A), as it has been previously reported (Simoes et al. 2011, Carvajal et al. 2019).

Overall *Wb* infection rate in *Ae. albopictus* from all the localities was of 40% (18/45; 37.9% of females, 43.7% of males; Table 2, Fig. 2A and B). Average *Wb* infection rate by locality was 13.3% and varied between localities (Table 2; Fig. 2E), with Tekik showing

Table 1. Primer nucleotide sequences used for amplification and typification of *Wolbachia* genome using DNA extracted from adult *Aedes albopictus* mosquitoes

Primer name	Nucleotide sequence (5′–3′)	PCR product size (bp)	*Wolbachia* strain	Reference(s)
16-2F	AGCTTCGAGTGAAACCAATTC	~1,000	Multistrain	Simoes et al. (2011)
16-2R	GAAGATAATGACGGTACTCAC			
328F	CCAGCAGATACTATTGCG	~300–400	A	Zhou et al. (1998); Ahmad et al. (2017)
691R	AAAAATTAAACG CTACTCCA			
183F	AAGGAACCGAAGTTCATG	~500–600	B	Zhou et al. (1998); Ahmad et al. (2017)
691R	AAAAATTAAACG CTACTCCA			

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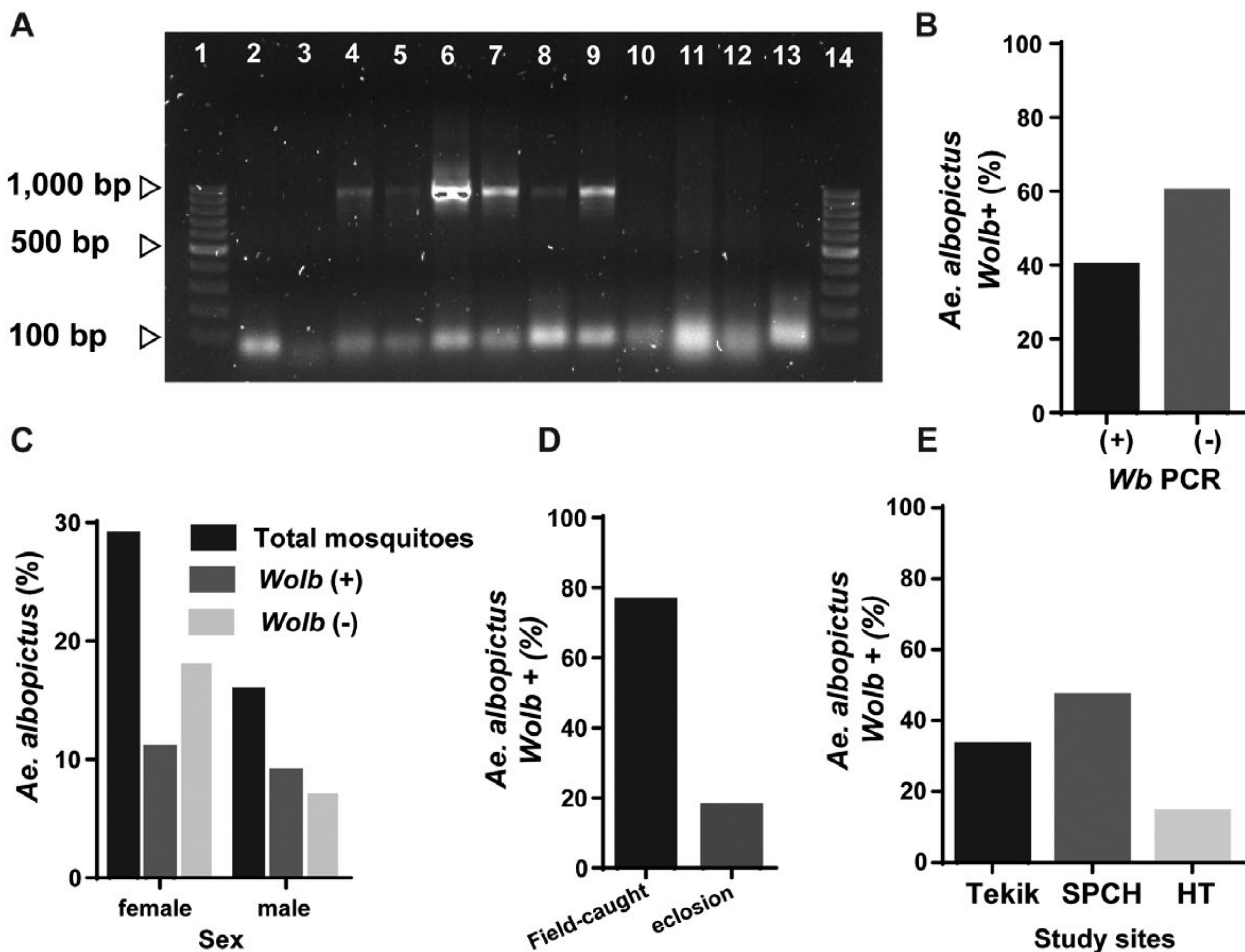

Fig. 2. Detection of *Wolbachia* infection in field-caught adult *Aedes albopictus* mosquitoes of the suburban areas of Merida, Yucatan. (A) PCR amplification of *Wb* DNA genome from total genomic DNA extracted from individual *Ae. albopictus* mosquitoes. A representative image showing a group of positive and negative samples (lanes 2–8). An amplicon of ~1,000 bp was considered positive for *Wb* infection (lanes: 4–9); positive control: genomic DNA from *Aedes aegypti* infected with *Wb* strain B as previously described (*w*MIDB; Xi et al. 2005; lane 9, female); negative control: genomic DNA from wild-type *Ae. aegypti* (*Wb* free) from Yucatan (*wt*MID) [lanes 10 (male)–11 (female)]; negative controls of PCR reaction mix without added genomic DNA (lanes 12–13). (B) Percentage of total *Wb* positive mosquitoes detected by PCR. (C) Sex distribution of *Wb* infection in *Ae. albopictus*. (D) Percentage of *Wb* infection detected in *Ae. aegypti* mosquitoes either adult field-caught or adult-reared under laboratory conditions from field-collected eggs. (E) Percentage of *Wb* infection and location of *Wb*-infected *Aedes albopictus* collected from the distinct localities in the suburban areas of Merida.

the highest percentage of *Wb* infection (>60%) of all mosquitoes tested in that site (33.3% males, 33.3% females; Fig. 2). The overall *Wb* infection rate (40%) found in this study lies between several field surveys around the world using field-collected specimens, which have found variable frequency rates of *Wb* infection ranging from 11 to 100% (Kitrayapong et al. 2002, de Albuquerque et al. 2011, Zhang et al. 2014, Noor Afizah et al. 2015, Ahmad et al. 2017, Guo et al. 2018, Martin et al. 2019, Hu et al. 2020).

A 76.5% (13/17) of all adult mosquitoes directly captured in the field were positive for *Wb* infection. However, only few adults of *Ae. albopictus* reared from field-collected eggs (17.8%, 5/28) showed the presence of *Wb* genome. *Wb* are intracellular bacterium that can be vertically transmitted from infected females to their offspring (transovarial transmission) and considered its primary mode of dissemination within host species (Werren et al. 2008). Particularly in eggs, the density of *Wb* infection can be affected under certain environmental circumstances such as high temperature or prolonged light exposure, which reduced the ability of *Wb* to invade and persist in the mosquito populations (Ross et al. 2019). Nevertheless, our results showed that vertical transmission of *Wb* occurs in wild populations of *Ae. albopictus* in the suburban areas of Merida.

Although the natural infection of wild *Ae. albopictus* with *Wb* was expected, so far only one single report exists describing the circulation of *Wb* in mosquitoes of Mexico. Roblero-Andrade et al. (2019) reported *Wb* infections from free-flying *Ae. albopictus* populations collected from cemeteries in Chiapas, Southeast of Mexico. In total, 42% (135/343) of females collected were positive for *Wb* infection; however, only one male mosquito was tested in the study and this was *Wb* negative. Additionally, large variability in the infection rates (7.7%–100%) was also observed and no identification of the infecting *Wb* strain was performed (Roblero-Andrade et al. 2019). Our study in Yucatan we found similar rates of overall *Wb* infection in total adults *Ae. albopictus* (40%) and total females (37.9%) screened; and we provide information on the *Wb* infection rate of male *Ae. albopictus* mosquitoes for the first time in Mexico.

Naturally occurring populations of *Ae albopictus* can be single-infected or coinfected with two types of *Wb* strains known as *w*AlbA (supergroup A) and *w*AlbB (supergroup B; Werren et al. 1995,

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Zhou et al. 1998). Here, a set of five mosquito samples were further analyzed by PCR to amplify a segment of the *wsp* gene to determine which strain of *Wb* was circulating in these populations of *Ae. albopictus* of Yucatan. We identified strains A and B as the infecting *Wb* endosymbiont of *Ae. albopictus* (Fig. 3A and B). All samples analyzed showed an amplification product with a molecular size lower than 400 bp corresponding to *Wb* strain A (Fig. 3A, lanes 2–6), and four of these samples (Fig. 3B, lanes 2–5) showed an amplification fragment for *Wb* strain B (approx. 500 bp). These results indicate that coinfection with *Wb* strains A and B occurs in *Ae. albopictus* of Yucatan.

As expected, the DNA used as positive control obtained from *Ae. aegypti* previously infected with *Wb* strain B (*w*MIDB) showed an amplification fragment for *w*AlbB, but not *w*AlbA (Fig. 3A and B, lane 8). No amplicon was obtained when genomic DNA obtained from the native *Ae. aegypti* strain (*wt*MID) was used (Fig. 2A, lanes 10–11). The lack of any amplification product confirmed that the native populations of *Ae. aegypti* mosquitoes in the localities included in the study are not hosting *Wb* strains.

Here, this study provides evidence of the circulation of *Wb* strains A and B in native populations of *Ae. albopictus* of Mexico. Further analyses using a bigger set of regionally/nationally field-collected mosquitoes, and the surveillance of *Wb* prevalence, as well as advanced molecular tools such as the multilocus sequence typing system or deep sequencing analyses of distinct *Wb* genes will help to better characterize the *Wb* strains that circulate in the populations of *Aedes* mosquitoes from Yucatan and Mexico. A better understanding of the distribution and diversity of *Wolbachia* in Mexico is valuable, as the infection of native populations of *Ae. albopictus* with *Wolbachia* can limit some arboviral infections, and it is also useful for the establishment of biological banks of locally occurring *Wb* strains that can be evaluated for the control of *Aedes*-transmitted diseases. Knowing the natural occurrence, infection frequency, and types of native *Wb* strains infecting both female and male *Ae. albopictus*—and other mosquito species—will provide a foundation for the design, implementation, and evaluation of *Wolbachia*-based interventions to control *Aedes* mosquitoes and ultimately, dengue, chikungunya, and Zika in Mexico.

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Table 2. *Wolbachia* infection of individual adult *Aedes albopictus* mosquitoes collected in distint suburban areas of Merida, Yucatan

Study sites	Sample type[a,b]	Sex (no. of individuals)			*Wolbachia* infection (no. of PCR + individuals)			*Wolbachia* infection rate (%)		
		F	M	Total	F	M	Total	F[c]	M[d]	Total[&]
Tekik	Free-flying adults from BG traps	3	1	9	3	1	6	33.3	33.3	13.3
	Adults emerged from eggs	0	5		0	2				
SPCH	Free-flying adults from BG traps	6	1	24	4	1	8	16.6	16.6	17.8
	Adults emerged from eggs	9	8		0	3				
HT	Free-flying adults from BG traps	6	0	12	4	0	4	33.3	0.0	8.9
	Adults emerged from eggs	5	1		0	0				
	Total	29	16	45	11	7	18	24.4	15.6	40

F, female; M, male; Tekik, Tekik de Regil; SPCH, San Pedro Chimay; HT, Hacienda Tahdzibichen.
[a]Field-collected free-flying adults (collected at BG traps).
[b]Field-collected adults emerged from eggs (collected in the field with ovitraps).
[c]Estimated from *Wolbachia* positive females or males and the total of mosquitoes captured per locality.
[d]Estimated from total of *Wolbachia* positive (female/male) mosquitoes and the total of mosquitoes tested.

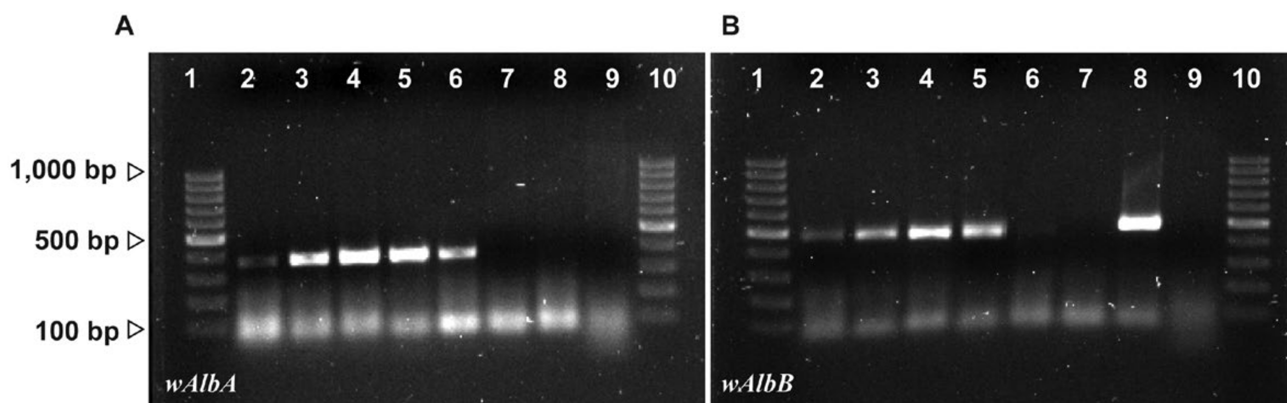

Fig. 3. Molecular characterization of *Wolbachia* strains infecting adult *Aedes albopictus* mosquitoes of the suburban areas of Merida, Yucatan. PCR amplification of *Wb* DNA genome from total genomic DNA extracted from individual *Ae. albopictus* mosquitoes using two set of primers specific for *Wb* strain A (A) and B (B). A representative image showing a group of five positive samples (lanes 2–7). An amplicon of 400 and 500 bp was considered positive for *Wb* infection with strain A and B, respectively; positive control: genomic DNA from *Aedes aegypti* infected with *Wb* strain B (*w*MIDB; Xi et al. 2005; lane 8); negative control: genomic DNA from wild-type *Ae. aegypti* (*Wb* free) from Yucatan (*wt*MID; lane 9). DNA marker of 100 bp (lanes 1 and 10).

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Author Contributions

Collection, taxonomic classification, maintenance, and rearing of mosquitoes: A.M.P., A.C.M., Y.C.P., S.P.C., and UCBE-LCB team. Conceived and designed the experiments: H.P.G., A.M.P., Y.C.P., S.P., and P.M.S. Performed the experiments: H.P.G., S.P.C., A.M.P., Y.C.P. Analyzed the data: H.P.G., A.M.P., Y.C.P., S.P.C., A.C.M., and the UCBE-LCB team. Contributed reagents/materials/analysis tools: P.M.S., G.T.A., and G.V.P.. Wrote the paper: H.P.G., A.M.P., Y.C.P., A.C.M., and P.M.S. The following names represent the active members of the UCBE-LCB team at UADY that significantly contributed to the development of this study: Jorge Palacio-Vargas (also at the Secretary of Health, Yucatan), Javier Pérez-Ojeda, Juan Navarrete-Carballo, Wilbert Bibiano Marin, and Anuar Medina Barreiro.

Conflict of Interest

The authors declare no conflicts of interest.

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