Natural coinfection with two *Wolbachia* supergroups in wild mosquitoes of *Aedes albopictus*

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Abstract

Objective. To understand, from an exploratory study, the diversity of *Wolbachia* strains present in species of the genus *Aedes* distributed in southern Mexico. **Materials and methods.** DNA was extracted from mosquitoes collected at 12 places and analyzed for the presence of *Wolbachia* by PCR using primers for wsp, 16S rRNA, ftsZ and groE genes. To determine the supergroup *Wolbachia* belongs, a phylogenetic analysis was performed with the wsp gene. A total of 639 mosquitoes of the genus *Aedes* were collected, 70.3% corresponded to *Aedes albopictus*, 11% for *Aedes quadrivittatus*. **Results.** The prevalence of *Wolbachia* in *Ae. albopictus* was 84% of all sampling sites, while for *Ae. quadrivittatus* corresponded to 79%. For *Ae. albopictus*, the coinfection between supergroups A and B, was confirmed while in *Ae. quadrivittatus* only supergroup B was detected. **Conclusion.** This study documents for the first time the presences of *Wolbachia* in species of the genus *Aedes albopictus* and *Aedes quadrivittatus*, in Chiapas Mexico.

Keywords: vectors; endosimbiont; supergroup A/B; arbovirus; *Aedes*

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Resumen

Objetivo. Comprender, a partir de un estudio exploratorio, la diversidad de cepas de *Wolbachia* presentes en *Aedes* distribuidas en el sur de México. **Material y métodos.** Se extrajo ADN de mosquitos colectados en 12 lugares y se analizó la presencia de *Wolbachia* por PCR utilizando primers para los genes wsp, 16S rRNA, ftsZ y groE. Para determinar el supergrupo al que pertenece *Wolbachia*, se realizó un análisis filogenético con el gen wsp. Se colectó un total de 639 mosquitos del género *Aedes*, 70.3% correspondió a *Aedes albopictus*. **Resultados.** La prevalencia de *Wolbachia* en *Ae. albopictus* representó 84% de todos los sitios de muestreo, mientras que para *Ae. quadrivittatus* correspondió a 79%. Para *Ae. albopictus*, se confirmó la coinfección entre los supergrupos A y B, mientras que en *Ae. quadrivittatus* sólo se detectó el supergrupo B. **Conclusión.** Este estudio documenta por primera vez la presencia de *Wolbachia* en *Ae. albopictus* y *Ae. quadrivittatus*, en Chiapas México.

Palabras clave: vectores; endosimbionte; supergrupo A/B; arbovirus; *Aedes*

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Mosquitoes (Diptera: Culicidae) encompasses species considered vectors of viruses such as dengue, Zika and chikungunya, which represent a serious public health problem. In Mexico the species *Aedes albopictus* and *Aedes quadrivittatus* are two species wide distributed. The Asian tiger mosquito *Aedes albopictus*, is currently distributed in 22 states of Mexico[1] and is considered an invasive species and secondary vector of arboviruses.[2,3] In the case of *Aedes quadrivittatus* its epidemiological role in pathogen transmission is unknown but has been recorded in 11 states.[4-6] This species is mainly found in forest and mountain areas with dense vegetation. This mosquito has been collected sharing breeding sites with *Aedes albopictus* in natural containers as well as artificial ones.[7]

Biological control measures include the use of the *Wolbachia* symbiont (Rickettsiales: Anaplasmataceae), an intracellular α-proteobacteria that is transmitted maternally in several species of arthropods and nematodes.[8] Worldwide, the presence of *Wolbachia* has been found naturally infecting *Ae. Albopictus*[9] with the strains *w*AlbA and *w*AlbB, which can be detected individually or in coinfection (superinfection) in Mexico, Asia and USA.[10-13] The presence of these two strains had been recorded that can induce cytoplasmic incompatibility (CI) between populations of *Ae. albopictus*, as well as increase fertility, longevity and favor the hatching of a greater number of eggs in females.[12,14]

Recently has been proposing that biological control for blocking arbovirus transmission is the introduction of the strain *w*Mel or *w*AlbB of *Wolbachia* in mosquitoes.[15,16] This bacterium develops in the mosquito cells, guaranteeing its propagation, stimulating the mosquito's immune system, causing alterations such as cytoplasmic incompatibility and decreasing the susceptibility to arbovirus infection within mosquitoes.[17]

Nevertheless, the role of *Wolbachia* strains in Mexican mosquitoes is not completely clear due to the few studies are available. Therefore the detection and typing of this endosymbiont associated with mosquitoes is highly relevant to continue evaluating its role as a biological control to decrease the virus transmission.[10] In particular, the area of the Soconusco, Chiapas, Mexico, is an especially important region for the country due it is a border area with Guatemala, therefore monitoring the populations of mosquitoes must be essential to create prevention strategies, and control for disease transmission in both countries. The objective of this work was performing an exploratory study to know the diversity of *Wolbachia* strains present in the mosquitoes *Ae. albopictus* and *Ae. quadrivittatus* in Chiapas, Mexico.

Materials and methods

Collection and species identification. The collection of mosquitoes was carried out in 12 places from the Soconusco, Chiapas, during rainy season: between August and October 2017. The sites selected were Huixtla (15°08'N and 92°28'W), Mazatán (14°51'N 92°26'W), Ahucatlán (17°54'N 98°43'W), Álvaro Obregón (14°56'N 92°23'W), Cacahoatán (14°59'N 92°09'W), Ciudad Hidalgo (14°41'N 92°10'W), Huehuetán (15°02'N 92°23'W), Tapachula (14°53'N 92°14'W), Río Florido (14°52'N 92°21'W), Tuxtla Chico (14°57'N 92°11'W) and Tuzantán (15°09'N 92°25'W) and Chiquihuites (15°05'N 92°06'W) (figure 1). The collected specimens were individually stored, dry at a temperature of -20 °C until processing. For the morphological identification of the collected species, specialized taxonomic keys were used.[18,19]

DNA extraction and PCR. For DNA extraction, the DNAzol kit (Invitrogen, San Diego, CA, USA) was used, following the supplier's instructions. *Wolbachia* detection was performed by amplifying a ≈590 bp fragment of the *wsp* gene using the oligonucleotides wsp81F (5'-TGG TCC AAT AAGTGA TGA AGA AAC-3') and wsp691R (5'-AAA AATTAA ACG CTA CTC CA -3 ').[20] The amplification conditions for *wsp* by PCR were: denaturation at 95 °C for 5 min, followed by 30 cycles of 94 °C, 1 min; 55 °C, 1 min and 72 °C 1 min; a final extension of 72 °C. Additionally, for the specific identification of supergroups A and B of *Wolbachia*, a duplex PCR was performed using primers 328F (5'-CCA GCA GAT ACT ATT GCG-3'), 183F (5'-AAG GAA CCG AAG TTC ATG-3 ') and 691R (5'-AAA AAT TAA ACG CTA CTC CA-3') using previously described conditions.[21]

Additionally, for *Wolbachia* typing, the 16S rRNA, ftsZ and groE genes were amplified; a ≈761bp fragment of 16S rRNA gene were amplified using the primers 16SFor (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 16SRev (5'-CCG TCA ATT CMT TTG AGT TT-3') with the following conditions 95 °C 4 min; followed by 30 cycles of 95 °C, 1 min; 52 °C, 1 min and 72 °C 1 min, a final extension of 72 °C, 10 min. ftsZ gene, used the primers FtsZFor (5'-ATY ATG GAR CAT ATA AAR GAT AG-3') and FtsZrev (5'-TCR AGY AAT GGA TTR GAT AT-3') which amplify ≈ 455pb, using a cycling 94 °C for 5 min; 30 cycles de 94 °C for 30 seg, 50 °C for 45 seg, 72 °C for 1:30 min and 72 °C, 10 min. Finally, for the amplification of a fragment of ≈ 630 bp of the groE gene, we used the primers groEwF (5'- GAA GAA AAA CAA GGT GGA ATT G-3') and GroEwR (5'-GTA CCA TCA CCA ACT TTG TC-3 ') using the conditions: 95 °C for 5 min; 30 cycles de 94 °C for 1 min, 48 °C for 1 min, 72 °C for 2 min and 72 °C for 5 min.

Phylogenetic analysis. The PCR products of the *wsp*, 16S rRNA, *groE* and *ftsZ* genes were directly sequenced using the labeled dideoxynucleotide method on the 3130 Genetic Analyzer sequencer (Applied Biosystems). The obtained electropherograms were visualized and edited in the Chromas software. Each sequence was compared with sequences available in the GenBank database using the Basic Local Alignment Search Tool (BLAST),* as a preliminary confirmation of the identity of each sequence. The DNA sequences were aligned with other *Wolbachia* reference sequences deposited in GenBank using MEGA version X. For the analysis of the obtained sequences, a Maximum Likelihood reconstruction was performed in MEGA X with a bootstrap of 1 000 repetitions, for all genes the Tamura 3-parameter substitution model (T92) + Gamma distribution. Genetic distances were calculated in MEGA X.

Results

Collection and identification of specimens. To typification of *Wolbachia*, 639 mosquitoes of the *Aedes* genus were collected, of which 459 (70.3%) corresponded to *Ae. albopictus*. The sites with the highest abundance were Tapachula, Huehuetán and Huixtla, with a percentage of 33.6%, 31.4% and 18.7%, respectively, the rest of the sites represented 0.6% to 4.3%. While *Ae. aegypti* (Linnaeus) was collected in 8.1%, *Ae. taeniorhynchus* (Wiedemann) (2.2%), *Ae. angustivittatus* Dyar & Knab (8.5%) and *Ae. quadrivittatus* (11%). This last species was found only in the town of Chiquihuites (figure 1).

Supergroup of Wolbachia *detection.* We analyzed all specimens for the presence of *Wolbachia* by duplex PCR of which only *Ae. albopictus* and *Ae. quadrivittatus* were positive for the presence of this endosymbiont (table I). The prevalence of *Wolbachia* in *Ae. albopictus* was 84%, reporting the highest prevalence in Huehuetán and Tapachula (table I). While the prevalence of *Wolbachia* in *Ae. quadrivitattus* was 79%, in Chiquihuites (figure 1). Supergroups A and B coinfections were detected for all *Ae. albopictus* specimens, while in *Ae. quadrivittatus* we only detected the presence of supergroup B. Only 20% of the positive specimens were selected for molecular typing of the *Wolbachia* strains using the *groE*, *ftsZ*, and 16S rRNA genes, and performed the subsequent sequencing and phylogenetic analysis.

Wolbachia *DNA detection and sequences analysis.* In *Ae. albopictus*, we amplified a fragment of ≈ 768 bp of the 16S rRNA gene (accession numbers: OM744347, OM744348, OM744349, OM744350, OM744351, OM744352, OM744353, OM744354), ≈455bp for the *ftsZ* gene (ac-

* http://blast.ncbi.nlm.nih.gov/Blast.cgi

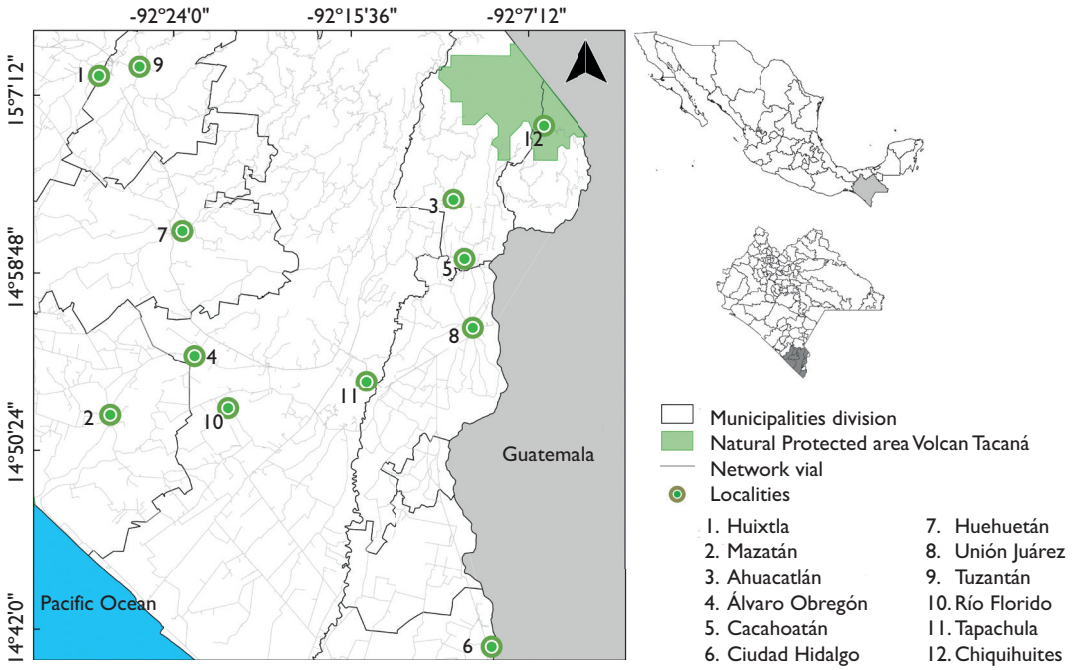

FIGURE 1. MAP OF THE GEOGRAPHIC LOCATION OF THE SITES SAMPLED DURIGN THIS STUDY IN SOCONUSCO, CHIAPAS, MEXICO BETWEEN AUGUST AND OCTOBER 2017

cession numbers: OM827058, OM827059, OM827060, OM827061, OM827062, OM827063, OM827064, OM827065), ≈531 bp for the *groE* gene (accession numbers: ON107284, ON107285, ON107286, ON107287, ON107288, ON107289, ON107290) and ≈550 bp for the *wsp* gene (accession numbers: OM914798, OM914800, OM914797, OM914801, OM914796, OM914799) to identify the presence of the endosymbiont *Wolbachia* in specimens of the genus *Aedes* collected from Chiapas, Mexico.

All the sequences obtained for *Ae. quadrivittatus* for all genes analyzed were 100% between them and represent the first record of the association with *Wolbachia* worldwide. The *ftsZ* gene (OQ512868, OQ512869, OQ512870) exhibited similarities of 99.79% with the sequence of *Wolbachia* endosymbiont of *Leptosia nina* from India (FJ392396.1). The *wsp* gene (accession numbers: OQ512864, OQ512865, OQ512866, OQ512867) exhibited similarities of 99.64% with the sequence of *Wolbachia* endosymbiont of *Bicyclus evadne* from Finland (KY658543.1). The 16S rRNA gene (OQ543113.1, OQ543114.1, OQ543115.1) exhibited similarities of 99.88% with the sequence of *Wolbachia* endosymbiont of *Aedes albopictus* from Pakistan (KX611380.1). The *groE* gene (accession numbers: OQ512871, OQ512872, OQ512873, OQ512874) exhibited similarities of 99.81% with *Wolbachia* endosymbiont of *Drosophila mauritiana* from USA (CP034335.1).

Respect to *Ae. albopictus*, the sequences obtained from the 16S rRNA gene showed similarities of 99.76% with sequences of *Wolbachia* endosymbiont of *Ae. albopictus* collected in Pakistan (KX611380.1) and 100% similarity with *Wolbachia* endosymbiot of *Culex pipiens* collected in Belgium (KJ512994.1). The *groE* gene showed 100% sequence similarity with *Wolbachia* endosymbiont of *Ae. albopictus* collected from China (CP041924.1) and 99.81% with another strain of *Wolbachia* detected in *Drosophila mauritiana* from the USA (CP031335.1). The *ftsZ* gene exhibited similarities of 98.74% with the sequence of the *Wolbachia* endosymbiont of *Spalangia cameroni* from the USA (KT121485.1). The *wsp* gene exhibited 100% similarities with a strain of *Wolbachia* detected in *Ae. aegypti* collected in India (MN307069.1), and *Wolbachia* detected in *Ae. albopictus* collected in China (CP041924.1) and Malaysia (MH418465.1).

According to the genetic distances, the sequences obtained in this study in the case of *Ae. albopictus* were identical to those of the wAlbA strain of *Wolbachia* endosymbionts of *Ae. albopictus* collected in India, Malaysia, China, and Taiwan (figure 2), confirming the presence of wAlbA strain for this study. In the same way, the genetic distances within supergroup B indicate that the sequences obtained from this study were identical to the wAlbB strain as that circulates in *Ae. aegypti* and *Ae. albopictus* from Malaysia, India, Russia, and China. In the phylogenetic tree the sequences of *Ae. albopictus* and *Ae. quadrivittatus* clustering in a cluster with bootstrap of 93%, but the genetic distances between this species is 0.5% and with other *Wolbachia* endosymbiont of the genus *Culex* 0.65%.

Table I
Detection of supergroups A and B of *Wolbachia* in *Aedes albopictus* and *Wolbachia* group B in *Aedes quadrivuttatus* females collected during August and October 2017, in Chiapas, Mexico

Locality	*Aedes albopictus*			*Aedes quadrivittatus*		
	collected	*wsp A/B (+)*	*% infection*	*collected*	*wsp B (+)*	*% infection*
Huixtla	84	55	65	0	0	0
Mazatán	4	2	50	0	0	0
Ahuacatlán	3	3	100	0	0	0
Álvaro Obregón	5	1	20	0	0	0
Cacahoatán	18	14	78	0	0	0
Ciudad Hidalgo	20	20	100	0	0	0
Huehuetán	141	133	94	0	0	0
Tapachula	151	140	93	0	0	0
Río Florido	15	11	73	0	0	0
Tuxtla Chico	13	1	8	0	0	0
Tuzantán	5	5	100	0	0	0
Chiquihuites	0	0	0	70	55	79
Total	459	385	84	70	55	79

FIGURE 2. PHYLOGENETIC ANALYSIS USING MAXIMUM LIKELIHOOD FOR A FRAGMENT OF THE WSP GENE OF *WOLBACHIA* DETECTED IN *AEDES ALBOPICTUS* AND *AEDES QUADRIVITTATUS*. THE OBTAINED SEQUENCES ARE MARKED WITH A CIRCLE, TRIANGLE AND SQUARE. MOSQUITOES WERE COLLECTED IN SOCONUSCO, CHIAPAS, MEXICO, BETWEEN AUGUST AND OCTOBER 2017

Discussion

The study of mosquito vectors of diseases in Mexico is of great relevance in public health, since it is the only way to be able to prevent and establish efficient control measures. In this study, we confirm the natural infection by *Wolbachia* in two species of mosquitoes of the genus *Aedes* from the Soconusco, Chiapas, Mexico.

In the state of Chiapas, the natural infection rate with *Wolbachia* in *Ae. albopictus* was recorded previously, although the phylogenetic analysis was not carried out.[13] Therefore, this study represents the first record of two *Wolbachia* strains associated to *Ae. albopictus*, and other one *Wolbachia* strain in *Ae. quadrivittatus* in Chiapas, Mexico. The strains were detected in *Ae. albopictus* were wAlbA and wAlbB in the 84% of the specimens

analyzed, this is the second time that these strains have been reported for Mexico, however the previous prevalence reported in the state of Chiapas and Yucatan were lower compared with this study.[13,22] Regarding *Ae. quadrivittatus* this is the first record of natural infection with *Wolbachia* worldwide, the strain detected belongs to supergroup B and was found with a prevalence of 79%, this strain represents a new record for this bacterium and was label as *wQua*B.

Although the *fts*Z, *groE*, 16S RNA genes, also allow efficient separation of supergroups A and B,[11,23] for this study these genes did not give enough phylogenetic information to separate the strains, this is probably because there are not enough sequences available for mosquitoes species (data not shown). Contrary, the *wsp* gene was very useful for typing *Wolbachia* strains because it has a big number of polymorphisms, singletons, and gaps, which allows us to observe a great amount of diversity between groups, unlike the other genes.[23,24] With the use of this gene, it was possible to confirm the coinfection between the *wAlb*A and *wAlb*B strains in 100% of the *Ae. albopictus* and the presence of *wQua*B in *Ae. quadrivittatus*.

Aedes albopictus has been reported with coinfection between the *wAlb*A and *wAlb*B strains, being of great relevance, since these strains can confer reproductive advantages by increasing fecundity, longevity and number of hatched eggs and therefore favour higher abundance of mosquitoes in some geographic areas.[11,12,14] This could be associated with the climatic variations of the sampling site, since it has been reported that the density of *Wolbachia* can be sensitive to these environmental changes.[10] Therefore, it is probable that the climatic characteristics of the Soconusco in Chiapas could favor the establishment of these two strains of *Wolbachia* in *Ae. albopictus*, which is favoring a great abundance of this species in the sampled sites. Furthermore, this highlights, is interesting since in other mosquito species the presence of two strains of *Wolbachia* is not very commonly recorded, and even less with a high prevalence, usually it is reported only one strain belong to one supergroup (A or B) but not both, since if the male and female possess two different strains of *Wolbachia* it is possible that this association may induce cytoplasmic incompatibility bidirectional (ICB).[14,25]

Regarding to *Ae. quadrivittatus* is an opportunistic species that had been recorded in areas with different degrees of disturbance in land use,[26,27] the highest abundances and dominance of this species is relates to the rainy season, and its considered a phytotelmata species, since its main breeding site are plants of the Bromeliaceae family and tree holes.[5,19] However, recent data report its presence in artificial containers, that could be sharing with other species adapted to urban environments. According with the feeding behavior is considered an opportunistic anthropophilic species.[7,28] Although, their medical relevance remains unknown for Mexico, in Panama had been isolated an unknown virus in this species.[29] More studies about virus detection in this species are necessaries in Mexico. This represents the first record of *Wolbachia* in this species, thus we do not know if this strain could cause reproductive alteration in this species.

In this study only female specimens were analyzed; thus, it is recommended evaluating the presence of this endosymbiont in male mosquitoes for both species. Since prevalence of *Wolbachia* in male mosquitoes could be important to assess the induce cytoplasmic incompatibility, representing an advantage for female specimens due they will be able to reproduce with infected and uninfected males.[14] This may be important for promote the incompatible insect technique (ITI), which in combination with the sterile insect technique (SIT) could cause generational blockage, thus demonstrating that it is a viable tool for vector mosquito control.[30] Additionally, we suggest evaluating whether the prevalence of virus in populations of *Ae. albopictus* is lower in the presence of superinfection with *Wolbachia* or not, in order to elucidate its possible biological control in this geographical site and confirm of discard the presence of viruses in *Ae. quadrivittatus* populations.

It is important to highlight the relevance of entomological monitoring for the study of species that play a role as vectors of pathogens. This study provides relevant information for the identification and typing of *Wolbachia* strains that infect *Ae. albopictus* and *Ae. quadrivittatus*, confirming that the distribution of this endosymbiont is broader than previously known, so it is suggested to carry out a search with a greater geographical range for this bacterium, since it could be of great epidemiological relevance for the possible decrease in transmission of viral diseases in Mexico.

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Author contribution

Yokomi N. Lozano-Sardaneta: research; resources, data analysis, supervision, manuscript writing, review, editing. Vicente Viveros-Santos: research; resources, data analysis, writing. Clemente Mosso-González: methodology, investigation, sampled, data analysis, writing. Armando Ulloa-García: investigation, writing. Jorge A. Torres-Monzón: methodology, research; resources, data analysis, supervision, manuscript writing, review, editing.

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

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