Proceedings of the Tenth International Conference on Urban Pests
Rubén Bueno-Marí, Tomas Montalvo, and Wm. H Robinson (editors) 2022
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BIOCONTROL OF ASIAN TIGER MOSQUITO, *AEDES ALBOPICTUS*, USING *WOLBACHIA* WPIP TO INDUCE INFERTILITY THROUGH CYTOPLASMIC INCOMPATIBILITY

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Abstract: *Aedes albopictus* was firsT reported in Valencia (Eastern Spain) in 2015. Once its establishment was confirmed, several mosquito management actions were applied in order to minimize its impact, including routinely larvicide treatments in public city catch basins, attendance of citizen complaints linked to species activity, private areas' inspections to eliminate domestic breeding sites, and campaigns of citizen awareness and environmental education. However, as in other affected Mediterranean cities, new innovative tools are needed to reduce nuisance and potential risks of arboviruses transmission. Among them, the use *Wolbachia pipientis* is considered a feasible approach. To reach this goal, several preliminary steps need to be performed, including (1) analysis of the presence of natural *Wolbachia* infection in local populations of *Ae. albopictus*; (2) establishment of a laboratory *Ae. albopictus* population free of *Wolbachia;* (3) characterization of *Wolbachia* wPip strains naturally infecting *Culex pipiens* in the Valencian area to identify the best strain to cause infertility due to cytoplasmic incompatibility (CI); (4) transfection of the *Wolbachia*-free *Ae. albopictus* population with selected Valencian *Wolbachia* wPip strains and determination of the CI effect when infected males mate with cured females. Here we present our latest achievements along the way to implement this strategy for *Ae. albopictus* suppression through massive release of males artificially infected with *Wolbachia* strains capable of inducing infertility due to CI.

Key words Incompatible Insect Technique (IIT), *Aedes albopictus*, *Culex pipiens*, Vector Management, Endosymbionts

INTRODUCTION

In recent years, climate change and globalization have favored the spread of insect species from tropical environments in our environment. This is the case of the Asian tiger mosquito *Aedes albopictus* (Skuse, 1894). It is present in the Valencian Community at least since 2015 (Bueno-Marí and Jiménez-Peydró, 2015), breeds in small pools of stagnant water, and the females need (almost exclusively) human blood to develop their eggs. Apart from the increase in cases of its annoying bites, it can transmit up to 22 infectious diseases (Paupy et al., 2009). Even though this are not yet frequent in our area (Bueno and Quero de Lera, 2021), surveillance measures are required, which necessarily involve the monitoring and control of mosquito populations.

Biological control of insect pests has spread as an alternative to the use of insecticides. One of these strategies is the use of *Wolbachia*, an alphaproteobacteria that naturally infects around 40% of arthropods and can cause alterations in their reproductive capacity (Werren et al., 2008). *Wolbachia* is an intracellular symbiont, lives mainly in the gonads and is transmitted from mother to offspring. Most strains cause reproductive disturbances in insects, including cytoplasmic incompatibility (CI) leading to embryo death. The alteration of the reproductive capacity of the insect host is actually caused by the presence of the WO bacteriophage virus integrated in the genome of more than

80% of the *Wolbachia* strains described (Bordenstein and Wernegreen, 2004). The main cause of this identified incompatibility is a toxin-antidote system encoded by the *cidA/cidB* operon in the prophage genome (Bonneau et al., 2018). If the proper antidote is not present in the egg before fertilization, the arrival of the toxin in the male gamete causes the destruction of the embryo.

Studies carried out on *Wolbachia pipientis* wPip, endosymbiont of the common mosquito *Culex pipiens* (Linnaeus, 1758), identified up to 17 haplotypes structured in five groups. There is compatibility between haplotypes of the same group, but incompatibility between groups and strains of different species. The five wPip groups present polymorphisms in the cidA/cidB genes, probably linked to the incompatibility among them (Bonneau et al., 2018). Incompatibility between *Wolbachia* strains from different species has been demonstrated. Thus, an wPip strain isolated from the common mosquito has a blocking effect when introduced into *Ae. albopictus* males (Calvitti et al., 2012). Furthermore, the male infected with wPip can effectively compete with males from the natural population for mating. The usefulness of this approach for the reduction of the tiger mosquito population in an urban environment has been recently confirmed (Caputo et al., 2020).

In view of the aforementioned background, we propose a similar strategy for the control of the tiger mosquito population in the city of València, using *W. pipientis* wPip isolated from common mosquitoes collected in the same city, as an absolutely biosafe strategy avoiding the use of pesticides and the introduction of foreign insects or bacteria.

MATERIALS AND METHODS

Maintenance and breeding of *Ae. albopictus* in the laboratory. A population of *Ae. albopictus* wAlb, kindly provided by Mara Moreno (Henkel Ibérica, S.A, Barcelona), is being maintained in the facilities of the Institute of Biodiversity and Evolutionary Biology (University of Valencia) in Paterna (València). Mosquitos are reared at 25 ± 2 ◦C, with of 60 ± 5% relative humidity and a photoperiod of 12:12 (Light: Dark) (Moreno-Gómez et al., 2021). In order to cure *Ae. albopictus* wAlb of *Wolbachia*, selected individuals were treated with tetracycline, following the procedure described by Dobson and Rattanadechakul (2001).

Molecular characterization of *Wolbachia* wPip substrains in València. Sampling and DNA extraction. *Cx. pipiens* larvae were collected in different ditricts (Table 1) and grown until adult stage in the facilities of Lokímica S.A. Individual adult insects were ground with a sterile plastic pestle and total DNA was purified with JetFlex Genomic DNA extraction Kit (Genomed, Germany).

Wolbachia *screening, genotyping and sequencing*. The detection of *Wolbachia* was based on the PCR amplification of partial sequences of the *wsp* and 16S rRNA genes with the KAPA2G Robust HotStart ReadyMix PCR Kit (Kapabiosystems, Boston, USA) with appropriated primer pairs and conditions (Carvajal et al., 2019; Braig et al., 1998). The determination of the wPip group was based on the PCR amplification of *pk*1 (Atyame et al. 2011) with VWR Taq DNA polimerasa, using the Key buffer provided by the supplier, with Mg^{2+} 2.5 mM and 4% DMSO. Amplicons automated Sanger sequencing was performed at the SCSIE sequencing facility of the University of Valencia. Read pairs were quality surveyed and assembled with Staden Package BLAST searches were performed to confirm the identity and group of the detected *Wolbachia* strain.

Phylogenetic analysis. Sequence alignments were conducted using ClustalW (Thompson et al., 1994) in MEGA v6 (Tamura et al. 2013). Phylogenetic analyses were performed using IQ-TREE (Nguyen et al., 2015) with the model HKY+F+G4. The *pk*1 sequences from the incompatibility groups were obtained from GenBank (PK1-I, AM397076.1; PK1-II, AM397077.1; PK1-III, AM397075.1; PK1-IV, AM397078.1; PK1-V, AM397079.1). *Wolbachia* of *Cimex lectularius* was used as outgroup (AP013028.1). We used FigTree v1.4.0 software to visualize and edit the phylogenetic tree.

RESULTS AND DISCUSSION

Since the detection of *Ae. albopictus* in the city of València, the municipal public health services have been deeply involved in the implementation of control strategies for nuisance reduction and minimization of potential arboviral transmission. They include insecticide treatment of breeding sites, community participation, and biological strategies. One of such biological strategies, the incompatible insect technique (IIT), does not require a mutation-causing treatment or genetic engineering of the insects, as it takes advantage of the existence of different *Wolbachia* strains belonging to different incompatibility groups. Very recent results have detected the natural presence of wild populations infected by the two common *Wolbachia* strains wAlbA and wAlbB in (unpublished results), known to be incompatible with *Wolbachia* wPip from *Cx. pipiens*, a species that is also widely spread in the city of València.

The work here presented is part of a global IIT project for the biocontrol of *Ae. albopictus* in València taking advantage of the local wPip substrains of *Wolbachia*. The initial steps of the global biocontrol strategy include the following specific objectives: (1) 1. Elimination of *Wolbachia* in *Ae. albopictus* raised in the laboratory, through an antibiotic treatment; (2) Characterization of the *Wolbachia* wPip substrains in natural populations of *Cx. pipiens* in the city of València; (3) Selection of the substrain(s) of interest based on cytoplasmic incompatibility (CI) with the autochthonous *Ae. albopictus* carriers of wAlbA and/or wAlbB. An aposymbiotic population of *Ae. albopictus* has been established and is being maintained in laboratory conditions. The absence of *Wolbachia* has been confirmed by PCR amplification of genes *wsp* and 16S rRNA, using the previously described protocols by Wiwatanaratanabutr (2013) and Simões et al. (2011), but increasing the number of cycles to 45 to improve sensitivity.

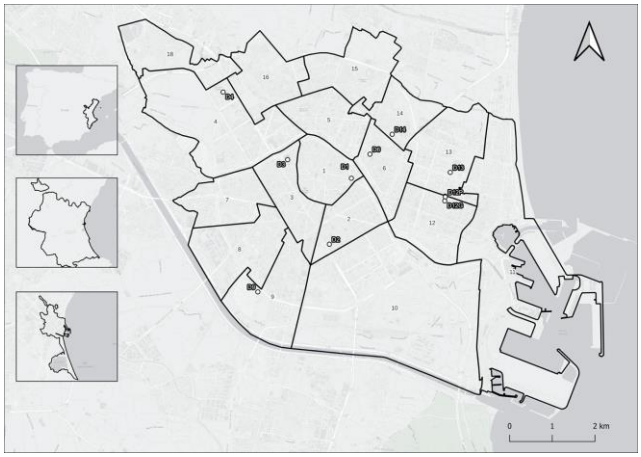

Figure 1. Map of València indicating the location of *Culex pipiens* samples analyzed.

The characterization of autochthonous *Wolbachia* wPip in local populations of *Cx. pipiens*, we have used the *pk*1 gene as a marker (Atyame et al., 2011). We analyzed 97 individuals (43 females and 54 males) from samples collected in several districts of València between May-October 2021 (Figure 1). We detected *Wolbachia* in 19 females and 25 males, (44% of analyzed individuals). All confirmed samples by amplicon sequencing were tested to detect their corresponding incompatibility group. Thirty five *pk*1 amplicons of about 1.3 kb in size were purified in sufficient quantity and quality for sequencing. The phylogenetic analysis of the 1279 informative nucleotides (Figure 2) indicate that most isolates belong to group III. Some isolated individuals show alternative haplotypes. The areas where they have been collected must be sampled to determine if these alternative haplotypes are punctual introductions from external sources or represent stable populations that could be used as a source of an alternative *Wolbachia* from a different incompatibility group.

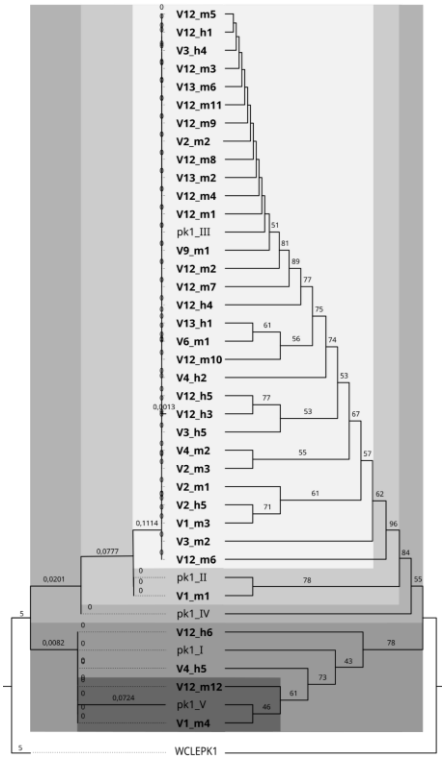

Figure 2. Phylogenetic analysis of the *Wolbachia* wPip substrains found in different populations of common mosquitoes in the city of València based on the *pk1* gene. On the left, the real phylogenetic distances are indicated; on the right, the dendrogram is shown for a better visualization of the phylogenetic relationships and the bootstrap values. Trees obtained with iqtree2 with the HKY+F+G4 model.

CONCLUSIONS

Based on our results, we will start the breeding and selecting adult males of *Ae. albopictus* transfected with wPip-VAL, to carry out laboratory tests to evaluate their competence capacity against autochthonous males and determine the capacity of the new strain to control tiger mosquito populations. In the near future, we intend to characterize the cidA/cidB operon of the WO prophage of which the selected *Wolbachia* wPip strains are carriers, to deepen in the knowledge of these toxin-antitoxin system, in order to search for alternative strains that could be reared separately in the laboratory and released choicely when the CI is lost due to the putative accidental release of females infected with one of the strains. The complete genome sequencing of the *Wolbachia* wPip strains of interest b their greater blocking effect, would help to understand this interesting phenotype in biocontrol strategies.

ACKNOWLEDGEMENTS

This work was supported by Ajuntament de València contract OTR2021-21502SERVI (E-02401-2021-000479-00).

REFERENCES CITED

Atyame, C.M., F. Delsuc, N. Pasteur, M. Weill, and O. Duron. 2011. Diversification of *Wolbachia* endosymbiont in the *Culex pipiens* mosquito. Mol. Biol. Evol. 28(10): 2761-2772.

Bonneau, M., C. Atyame, M. Beji, F. Justy, M. Cohen-Gonsaud, M. Sicard and M. Weill. 2018. *Culex pipiens* crossing type diversity is governed by an amplified and polymorphic operon of *Wolbachia*. Nat. Commun. 9: 319

Bordenstein, S.R. and J.J. Wernegreen. 2004. Bacteriophage flux in endosymbionts (*Wolbachia*): infection frequency, lateral transferand recombination rates. Mol. Biol. Evol. 21: 1981–1991.

Braig, H.R., W. Zhou, S.L. Dobson and S.L. O'Neill. 1998. Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. J. Bacteriol. 180: 2373–2378.

Bueno-Marí, R. and R. Jiménez-Peydró. 2015. First observations of homodynamic populations of *Aedes albopictus* (Skuse) in Southwest Europe. J. Vector Borne Dis. 52: 175–177.

Bueno-Marí, R. and F. Quero de Lera. 2021. Gestión vectorial de los casos de arbovirosis notificados en la ciudad de València, España (2016-2018). Revista Española de Salud Pública, 95: 1–12.

Calvitti, M., R. Moretti, A.R. Skidmore and S.L. Dobson. 2012. *Wolbachia* strain wPip yields a pattern of cytoplasmic incompatibility enhancing a *Wolbachia*-based suppression strategy against the disease vector *Aedes albopictus*. Parasit. Vectors 12: 254.

Caputo, B., R. Moretti, M. Manica, P. Serini, E. Lampazzi, M. Bonanni, G. Fabbri, V. Pichler, A. della Torre and M. Calvitti. 2020. A bacterium against the tiger: preliminary evidence of fertility reduction after release of *Aedes albopictus* males with manipulated *Wolbachia* infection in an Italian urban area. Pest Manag. Sci. 76: 1324–1332.

Carvajal, T.M., K. Hashimoto, R.K. Harnandika, D.M. Amalin and K. Watanabe. 2019. Detection of *Wolbachia* in field-collected *Aedes aegypti* mosquitoes in metropolitan Manila, Philippines. Parasit. Vectors 12: 361.

Dobson, S.L. and W. Rattanadechakul. 2001. A novel technique for removing *Wolbachia* infections from *Aedes albopictus* (Diptera: Culicidae). J. Med. Entomol. 38: 844–849.

Moreno-Gómez, M., R. Bueno-Marí and M.A. Miranda. 2021. A three-pronged approach to studying sublethal insecticide doses: characterising mosquito fitness, mosquito biting behaviour and human/environmental health risks. Insects, 12: 546.

Nguyen, L.T., H.A. Schmidt, A. von Haeseler and B.Q. Minh. 2015. Q-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol. Biol. Evol. 32: 268–274.

Paupy, C., H. Delatte, L. Bagny, V. Corbel and D. Fontenille. 2009. *Aedes albopictus*, an arbovirus vector: from the darkness to the light. Microbes Infect. Dec; 11(14-15):1177-85.

Simões P.M., G. Mialdea, D. Reiss, M.F. Sagot, S. Charlat. 2011. *Wolbachia* detection: an assessment of standard PCR protocols. Mol. Ecol. Resour. 11: 567–572.

Tamura, K., G. Stecher, D. Peterson, A. Filipski, S. Kumar. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30: 2725–2729.

Thompson, J.D., D.G. Higgins and T.J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673–4680

Werren, J.H., L. Baldo and M.E. Clark. 2008. *Wolbachia*: Master manipulators of invertebrate biology. Nat. Rev. Microbiol. 6: 741–751.

Wiwatanaratanabutr, I. 2013. Geographic distribution of wolbachial infections in mosquitoes from Thailand. J. Invertebr. Pathol. 114: 337–340.