REVISTA ESPAÑOLA DE SALUD PÚBLICA
RESP
Issue
97
3/2/2023

Pilot survey of Wolbachia pipientis infections in populations of Aedes albopictus in the city of València (Spain): implications for mosquito control

AUTHORS

Rubén Bueno-Marí	(1,2)	[ORCID: 0000-0002-4898-8519]
Rebeca Domínguez-Santos	(3)	[ORCID: 0000-0001-5158-6695]
María Trelis	(2,4)	[ORCID: 0000-0002-5977-3337]
Emilio Garrote-Sánchez	(3)	[ORCID: 0000-0003-2345-3179]
María Cholvi	(2)	[ORCID: 0009-0009-1275-3951]
Fermín Quero de Lera	(5)	
Messaoud Khoubbane	(2)	[ORCID: 0000-0003-4803-7198]
Antonio Marcilla	(2,4)	[ORCID: 0000-0003-0004-0531]
Rosario Gil	(3,6)	[ORCID: 0000-0002-9397-1003]

AFFILIATIONS

(1) Departamento de Investigación y Desarrollo (I+D), Laboratorios Lokímica. Paterna (València). España.
(2) Área de Parasitología, Departamento de Farmacia y Tecnología Farmacéutica y Parasitología, *Universitat de València*. Burjassot (València). España.
(3) Instituto de Biología Integrativa de Sistemas (I2SysBio), *Universitat de València*/CSIC. Paterna (València). España.
(4) Unidad Mixta de Endocrinología, Nutrición y Dietética Clínica, Instituto de Investigación Sanitaria La Fe. València. España.
(5) Servicio de Sanidad y Consumo, Ayuntamiento de València. València. España.
(6) Área de Genómica y Salud, Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Com. Valenciana (FISABIO). València. España.

ORIGINAL
Received: 9/30/2022
Accepted: 2/13/2023
Published: 3/2/2023
e202303017
e1-e15

Infecciones por Wolbachia pipientis en poblaciones de Aedes albopictus en la ciudad de València (España): implicaciones para el control de mosquitos

Authors declare that there is no conflict of interests.

CORRESPONDENCE

Rubén Bueno Marí
Parque Tecnológico de Paterna. Ronda Auguste y Louis Lumiere, 23, nave 10. CP 46980. Paterna (València). España.
rbueno@lokimica.es / ruben.bueno@uv.es

Rosario Gil
I2SysBio. Parque Científico de la *Universitat de València*. C/ Agustín Escardino, 9. CP 46980. Paterna (València). España.
Rosario.gil@uv.es

SUGGESTED CITATION

Bueno-Marí R, Domínguez-Santos R, Trelis M, Garrote-Sánchez E, Cholvi M, Quero de Lera F, Khoubbane M, Marcilla A, Gil R. Pilot survey of *Wolbachia pipientis* infections in populations of *Aedes albopictus* in the city of València (Spain): implications for mosquito control. Rev Esp Salud Pública. 2023; 97: March 2nd e202303017.

ABSTRACT

BACKGROUND // The presence of *Aedes albopictus*, of high sanitary and social impact, was first reported in València (eastern Spain) in 2015. Innovative tools for its control include the use of the endosymbiotic bacterium *Wolbachia pipientis*. The release of male mosquitoes infected with the *w*Pip strain, has proven very promising for large-scale Incompatible Insect Technique (IIT) applications. Before this strategy can be implemented in València, it is important to know whether the natural local mosquito populations are *Wolbachia*-infected and, if so, identifying the infecting strains/supergroups.

METHODS // Eggs were collected from the 19 districts of the València city between May and October 2019. A total of 50 lab-reared adult *Ae. albopictus* individuals were processed and analyzed for *Wolbachia* detection and molecular characterization. These actions took place within the framework of a collaboration established with the Department of Health and Consumers of the city council of València.

RESULTS // Our study revealed that 94% of the analyzed samples were naturally infected with *Wolbachia*. Both *w*AlbA and *w*AlbB supergroups were identified, with most samples (72% of the infected ones) carrying co-infections.

CONCLUSIONS // These data provide the first characterization of the *Wolbachia* presence in natural populations of *Ae. albopictus* in the Mediterranean area of Spain. This information is relevant to evaluate the potential use of *Wolbachia* strains in order to achieve the suppression of the Asian tiger mosquito populations through massive release of artificially infected males.

KEYWORDS // *Aedes albopictus*; *Wolbachia*; València; Insect Incompatible Technique (IIT); Biological control; Mosquito control; 16S rRNA gene; *wsp*.

RESUMEN

FUNDAMENTOS // La presencia de *Aedes albopictus*, de alto impacto sanitario y social, se informó por primera vez en Valencia en 2015. Las herramientas innovadoras para su control incluyen el uso de la bacteria endosimbiótica *Wolbachia pipientis*. La liberación de mosquitos machos infectados con la cepa *w*Pip ha demostrado ser muy prometedora para aplicar la Técnica de Insectos Incompatibles (IIT) a gran escala. Antes de que esta estrategia pueda implementarse, es importante saber si las poblaciones locales de mosquitos silvestres están infectadas por *Wolbachia* y, de ser así, identificar las cepas/supergrupos infectantes, siendo estos los objetivos del presente trabajo.

MÉTODOS // Se recolectaron huevos de los diecinueve distritos de València entre mayo y octubre de 2019, y se mantuvieron en el laboratorio hasta llegar a adultos. Un total de cincuenta individuos adultos de *Ae. albopictus* fueron procesados y analizados para detectar la presencia de *Wolbachia* y su caracterización molecular. Estas acciones se enmarcaron en la colaboración establecida con la Concejalía de Salud y Consumo del Ayuntamiento de València. La prueba exacta de Fisher fue utilizada para detectar la significación estadística de las diferencias entre grupos.

RESULTADOS // El 94% de las muestras analizadas estaban infectadas de forma natural con *Wolbachia*. Se identificaron los supergrupos *w*AlbA y *w*AlbB, y la mayoría de las muestras (72% de las infectadas) presentaban coinfecciones.

CONCLUSIONES // Los datos proporcionan la primera caracterización de la presencia de *Wolbachia* en poblaciones naturales de *Ae. albopictus* en el área mediterránea de España. Esta información es relevante para evaluar el potencial uso de cepas de *Wolbachia* de cara a la supresión de poblaciones de mosquito tigre asiático mediante la liberación masiva de machos infectados artificialmente.

PALABRAS CLAVE // *Aedes albopictus*; *Wolbachia*; València; Técnica de Insectos Incompatibles (IIT); Control biológico; Control de mosquitos; Gen de rRNA 16S; *wsp*.

INTRODUCTION

THE INVASION OF *AEDES ALBOPICTUS* (Skuse, 1894), known as Asian tiger mosquito, has been reported worldwide [1]. Its recognized clinical importance lies in its potential to transmit several pathogens causing diseases like dirofilariasis and different exotic anthroponotic arboviruses including dengue (DENV), chikungunya (CHIKV), or Zika fever (ZIKV) [2-5]. The increased risk of transmission is due to several concomitant reasons, including climatic and socioeconomic factors, international travel and trade, and public health systems [6]. In this context, Mediterranean countries such as Italy, France, Croatia and Spain have declared cases of autochthonous DENV, CHIKV and ZIKV transmission in recent years [5,7-10]. Among them, the situation of DENV is particularly worrying since dengue has emerged as the most important viral mosquito-borne disease globally in recent years [11]. In Southern Europe autochthonous dengue transmission episodes have occurred on several occasions [12], with a first European detection of *Ae. albopictus* mosquitoes infected with DENV in Spain in 2015 [13]. Traditional control measures to reduce mosquito populations include source reduction, public education, and insecticide application, routinely implemented by hundreds of municipalities across Europe [14]. However, in some cases, success may be limited due to low levels of community participation, lack of coordination between different administrations and poor practices in the application of insecticides. Moreover, insecticide resistance cannot be discarded as an additional problem to achieve optimal results on *Ae. albopictus* control in Europe [15]. Therefore, there is a great consensus among the scientific community about the need to search for innovative and complementary *Ae. albopictus* control methods in urban environments.

Ae. albopictus was first reported in Spain in 2004 [16]. Since then, it has spread from Catalonia to the rest of the Mediterranean area including the Balearic Islands [17], as well as other inland and northern regions in Spain such as Madrid, Extremadura and Basque Country [18-20]. In 2015 it was reported for the first time in the city of Valencia [21]. Thenceforth, its control has become a major goal for municipal public health services [22]. Major control measures include treatment with insecticides of breeding places, community participation, as well as biological strategies. The latter overcome some of the drawbacks of insecticide use, namely lack of specificity against certain insects, toxicity to humans and the environment, as well as the possibility of developing resistance [6,23].

Different biological strategies have been used worldwide [24], such as the application of biolarvicides mostly based on *Bacillus thuringiensis* formulates, the release of radiated males (Sterile Insect Technique, SIT), insect transgenesis techniques, or the use of endosymbionts like *Wolbachia* to cause the death of the embryos due to cytoplasmic incompatibility (CI) between the parental gametes (Incompatible Insect Technique, IIT) [25,26]. IIT has the advantage that it does not involve the release of mutant or genetically manipulated organisms. Population suppression of *Aedes* mosquitoes through the implementation of a *Wolbachia* strategy is considered a high impact method for insecticide resistance management [27].

Wolbachia pipientis (Alphaproteobacteria: Rickettsiales) is a maternally transmitted endosymbiont that is estimated to be able to infect up to 66% of known insect species [28]. This single species of the genus *Wolbachia* is classified into supergroups A to U [29]. Supergroups A and B infect only arthropods, where they act mostly as reproductive parasites. In these cases, distinct *Wolbachia* strains cause different alterations in their hosts to increase their transmission to the next generation, with CI as the most frequent manipulation [30]. *Wolbachia* has been used for pest

and disease vector control because it not only alters host reproduction, but also blocks virus replication and transmission (31). For this reason, endosymbiont-infected populations are being released into the field for two main purposes: population re-placement and population suppression (32). The success of both approaches relies on the ability of the new infecting *Wolbachia* strain to induce CI in the native mosquito population. Thus, the presence of some *Wolbachia* infections in wild mosquito populations might interfere with disease control programs, making population replacement or suppression challenging or even impossible.

The presence of *Wolbachia* strains *w*AlbA and *w*AlbB, belonging to the A and B supergroups respectively (33,34), has been widely described in natural populations of *Ae. albopictus*, where infected individuals usually carry one or both strains (35). No other strains have been found naturally in this mosquito species, although it cannot be discarded, since *Wolbachia* is known to undergo horizontal transmission between phylogenetically distant insect species (36). Pioneering studies performed in Italy, described the generation in the laboratory of a new *Ae. albopictus* AR*w*P line in which the natural *Wolbachia* strains have been replaced by the *w*Pip strain obtained from *Culex pipiens* (Linnaeus, 1758) (37); very recently, the same group reported the first field experiment in Europe to assess the incapacity of the male mosquitoes of the AR*w*P line to produce viable offspring after mating with wild *Ae. albopictus* females (38). These studies provide further support to the IIT as a suitable approach to reduce and/or suppress *Ae. albopictus* populations in the Mediterranean area.

In the present study, as a first step towards the implementation of an IIT strategy similar to the successful one mentioned above, we have investigated the presence of *Wolbachia* in natural populations of *Ae. albopictus* in the city of València (Spain).

MATERIALS AND METHODS

Mosquito sampling. Eggs were collected using standard ovitraps (9). Black plastic bowls (0.4 L volume) filled with water (2/3 of capacity) and supplemented with a wooden stick as oviposition support were used. Sampling was carried out in 2019, between May and October, coinciding with most relevant activity period of the species, in the 19 districts of the city of València, Spain [Figure 1; Annex I]. Sampling was spatiotemporally done as follows: May, urban center districts 1, 2 and 3; June, bordering urban districts 4, 5 and 6; July, bordering urban districts 7, 8 and 9; August, bordering urban districts 10, 11 and 12; September, bordering urban districts 13, 14, 15 and 16; October, peripheral rural districts 17, 18 and 19. Number of eggs collected by ovitraps per district ranges from 9 to 112. Specific microhabitats where ovitraps were installed are characterized by shady places well covered by vegetation. Field-collected eggs were reared under laboratory conditions until adult emergence, as previously described (39). Seven to ten days' adult insects, both male and female, were frozen at -20°C to kill them and were maintained in the freezer until total DNA extraction. Species identification and sex separation was confirmed under binocular microscope according to Schaffnet *et al.* criteria (40). The selection of individuals for *Wolbachia* analyses was done randomly by choosing at least 1 male and 1 female per district.

Wolbachia genotyping: DNA extraction, amplification, sequencing and phylogenetic analysis. Individual adult mosquitoes were lysed and homogenized in 200 µL of phosphate buffered saline (PBS) with the aid of sterile toothpicks. After centrifugation at low speed (800xg) for 20 min, total genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol, and using a final elution volume of 100 µL. The quality and quantity of DNA was determined with a NanoDrop ND-1000 spectrophotometer.

Figure 1
Spatial distribution of the sampling sites for collecting *Ae. albopictus* eggs in the 19 districts of the city of València, Spain (in numbers).

Dots indicate sampling points.

All tDNA samples were screened for the presence of *Wolbachia* based on polymerase chain reaction (PCR) amplification of the molecular markers *wsp* (primers: 81F, 5'-TGGTCCAATAAGTGATGAAGAAAC-3'; 691R, 5'-AAAAATTAAACGCACTCCA-3'), and 16S rRNA gene (primers: WolbF, 5'-GAAGATAATGACGGTACTCAC-3'; WspecR, 5'-AGCTTCGAGTGAAACCAATTC-3') **(41)**. Negative samples were tested with primers to amplify host ribosomal S7 gene to test DNA quality (5'ATGGTTTTCGGATCAAAGGT-3' and 5'-CGACCTTGTGTTCAATGGTG-3') **(42)**. Positive samples were then analyzed with forward *wsp*-primers specific for strains *w*AlbA (328F, 5'-CCAGCAGATACTATTGCG-3') and *w*AlbB (183F, 5'–AAGGAACCGAAGTTCATG-3'), together with the above mentioned reverse primer 691R **(35)**. Briefly, PCR amplifications were performed on 2-5 µL of insect's total DNA in 50 µL of reaction with 0.2 µM of each appropriate primer 1X Key Buffer, 2.5 mM MgCl2, 0.2 mM of each dNTP, 1 unit of Taq DNA Polymerase (VWR) and PCR-grade water up to final volume. 4% DMSO was added to the mix when the first PCR reaction failed. The thermal cycling profile was as follows: initial denaturation at 95 °C for 4 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, plus a final extension step of 10 min at 72 °C. The PCR amplification process was performed on two replicates for each sample, and all PCR experiments included positive and negative controls. As a positive control, we used tDNA of an *Ae. albopictus* line coinfected with *Wol-*

bachia *w*AlbA and *w*AlbB, kindly provided by Drs. Nuria Busquets and Sandra Talavera (Universitat Autònoma de Barcelona, Spain). In the negative control, water was added to the PCR reaction instead of tDNA. The amplicons size was checked by 2% agarose gel electrophoresis, stained with SafeView™ Classic stain (NBS Biologicals, Huntingdon, Cambridgeshire, UK), and visualized with UV light.

ABI sequencing was performed on selected amplified products at the sequencing facility of the Universitat de València (Servicio Central de Soporte a la Investigación Experimental, SCSIE) to confirm their identity. Sequencing reads were quality surveyed and assembled using the Staden Package (*http://staden.sourceforge.net*) (43).The obtained sequences were compared with *Wolbachia* sequences already available in GenBank through BLAST searches (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*) (44). The DNA sequences determined in this study have been deposited in GenBank (see Accession numbers in **Table 1**). Selected *Wolbachia* sequences available in GenBank **[Table 1]** were used to perform a sequence alignment with MEGA (45). Phylogenetic analysis was per-formed by maximum likelihood with GTR+G+I Model. Bootstrap analysis was performed with 1,000 replications. The FigTree v1.4.0 software (*http://tree.bio.ed.ac.uk/software/figtree/*) was used to visualize and edit the phylogenetic tree.

RESULTS AND DISCUSSION

OUR DATA REVEALED THAT 94% OF THE analyzed samples were naturally infected with *Wolbachia*, 72.3% of which were superinfected with strains *w*AlbA and *w*AlbB **[Table 2, Annex I]**. We searched for the presence of *Wolbachia* in 50 adult insects (25 females and 25 males) by PCR, using generic *wsp* primers. Of these, 47 samples (24 females and 23 males) tested positive, revealing a very high infection rate. The possibility of negative results due to an amplification problem caused by a low quality of purified tDNA was ruled out, as all three negative samples were positive for the host ribosomal S7 gene. Since co-infection with *w*AlbA and *w*AlbB strains has been frequently described worldwide (33), the positive samples were subsequently tested with strain-specific primers in separate reactions (35). All amplified and sequenced samples were identical to the *wsp* sequences of *w*AlbA and *w*AlbB found in GenBank, and grouped with the corresponding sequences in a phylogenetic analysis **[Figure 2]**.

The results obtained in our study confirm the possibility of implementing the IIT based on the release of *Ae. albopictus* males infected with *w*Pip in the city of Valencia in a near future, since the wild populations of Asian tiger mosquito are not naturally infected by this endosymbiont strain. Our next steps will focus on the selection of appropriate substrains of *w*Pip naturally infecting the autochthonous *Cx. pipiens* populations, and transfer them by egg microinjection to *Ae. albopictus*, to obtain and maintain in captivity an artificial colony whose males could be released into the field and cause CI after mating with wild females.

Recent research in Italy supports the release of males of a *Wolbachia* *w*Pip-infected *Ae. albopictus* line (AR*w*P) for autocidal suppression strategies against the Asian tiger mosquito (51,52). Before a similar initiative could be implemented in a new territory, the detection and identification of the *Wolbachia* strains present in local wild *Ae. albopictus* populations is convenient to prevent unexpected effects such as inefficient loss of compatibility (53).

This high rate of *Wolbachia* infection in a wild population of *Ae. albopictus* (94.0%) is similar to the prevalence found in Asian countries like China (93.3 and 95.52%) (54,55), Thailand (100%) (56), or Korea (99%) (57), as well as in the US (95%) (58), and Brazil (99,3%) (59), and far from the low prevalence found in Mexico (a median of 38%) (60). ▶

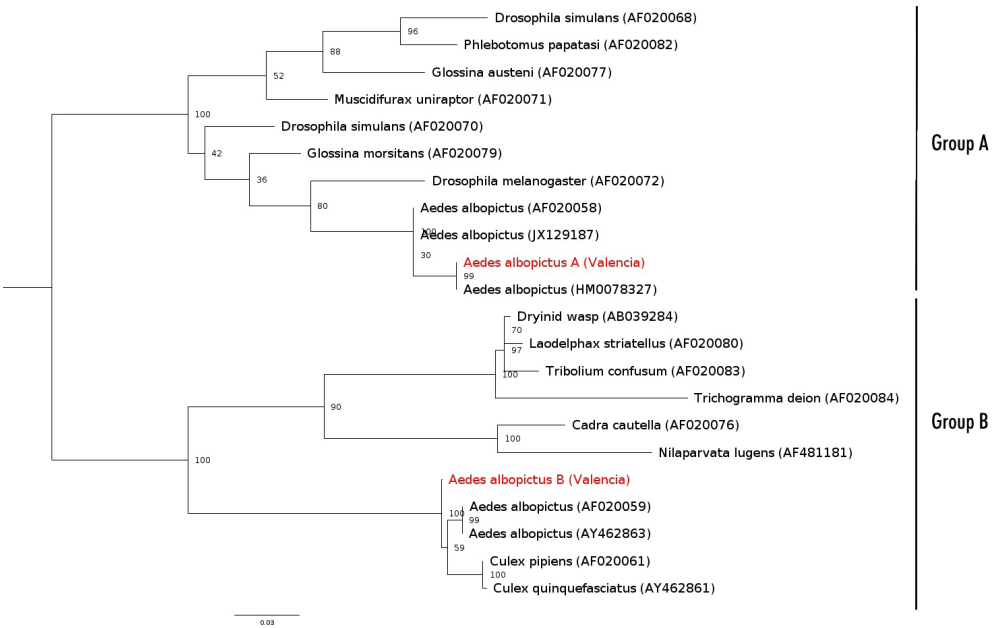
Table 1
Wolbachia partial *wsp* sequences included in the phylogenetic analysis.

Accession number	Insect host	Reference
AF020058	*Aedes albopictus*	33
AF020059	*Aedes albopictus*	33
AY462863	*Aedes albopictus*	44
HM007832	*Aedes albopictus*	45
JX129187	*Aedes albopictus*	45
OP066400	*Aedes albopictus*	This study
OP066401	*Aedes albopictus*	This study
AF020061	*Culex pipiens*	33
AY462861	*Culex quinquefasciatas*	44
AF020072	*Drosophila melanogaster*	33
AF020068	*Drosophila simulans*	46
AF020070	*Drosophila simulans*	46
AB039284	*Dryinid wasp*	47
AF020076	*Ephestia cautella*	33
AF020077	*Glossina austeni*	33
AF020079	*Glossina morsitans*	33
AF020080	*Laodelphax striatellus*	33
AF020071	*Muscidifurx uniraptor*	33
AF481181	*Nilaparvata lugens*	48
AF020082	*Phlebotomus papatasi*	33
AF020083	*Tribolium confusum*	33
AF020084	*Trichogramma deion*	33

Table 2
Infection status of the individual adult *Aedes albopictus* mosquitoes analyzed in this study.

Sex	A+B+	A+B-	A-B+	A-B-
Females	21	3	0	1
Males	13	1	9	2

Figure 2
Phylogenetic analysis of the *wsp* partial nucleotide sequences of *Wolbachia* obtained from *Ae. albopictus* collected in València, Spain (in red).



Scale bar represents substitutions per site.

Very few reports have been published in the Mediterranean countries, with large differences found in different locations in France (metropolitan and Corsica) **(53,61)**, and Greece **(53)**.

In our survey, the infectious status of males and females differed significantly (p-value 0.0067 using Fisher's exact test). Most infected mosquito females carried superinfections (84%); in the case of males, 54% of them carry also both *Wolbachia* strains, but 36% of them present single infection with *w*AlbB. These results are in accordance with what had been found previously, with superinfections virtually fixed among females **(33,35,56)**, and loss of *w*AlbA with age in males **(53)**.

A recent study using AR*w*P infected males and released in urban areas of Rome (Italy) have confirmed the feasibility of IIT as a way of controlling *Ae. albopictus* populations **(38)**. The authors presented promising results, as 30% of females collected in the release spots were sterile, and 20% had strongly reduced fertility compared with controls. Furthermore, male longevity is not affected by *w*Pip infection in the AR*w*P line, as they survived up to 2 weeks after release, which is considered adequate for the preservation of reproductive fitness in males, and very similar to wild Asian tiger mosquito males in the field under normal conditions **(62)**. These results are in the same line that those found in a previous laboratory study by the same group **(63)**, ▶

where no differences were found between uninfected and ARwP *Ae. albopictus* males regarding longevity, mating rate, sperm capacity and mating competitiveness in laboratory condition and greenhouses.

Finally, it is important to address the status of the regulatory aspects of using *Wolbachia*-based control approaches in Europe. Any product to be used to control unwanted organisms (including mosquitoes) that are harmful to human or animal health, or to the environment, or simply can cause damage to human activities, should be registered in Europe as a biocide according to the *EU Biocidal Products Regulation 528/2012* **(66)**. After collecting information about the use of *Wolbachia* as a potential biocide in Europe, the European Commission has recently approved its use stating as follows: "*The bacteria of the genus Wolbachia, or any preparation containing those bacteria, used for the purpose of inoculating those bacteria into mosquitoes, with the objective of creating non-naturally infected mosquitoes for vector control purposes, shall be considered a biocidal product; while non-naturally infected mosquitoes, regardless of the infection technique used, shall be considered neither a biocidal product nor a treated article*" **(65)**. Considering these regulations, there is a clear route now for the introduction process of *Wolbachia* into the vector control programs of Europe according to European laws. In this context, the València City Council is supporting an initiative regarding the implementation of *Wolbachia* strains for the control of *Ae. albopictus* populations which will be pioneering in Spain.

ACKNOWLEDGEMENTS

Authors would like to thank to technicians of Lokímica SL for their collaboration in the analysis and field collection of samples, respectively. Dr. Nuria Busquets and Dr. Sandra Talavera, from Animal Health Research Center IRTA-CReSA (Barcelona, Spain) are acknowledged for providing guidance and positive control samples. This work was carried out within the framework of AIM-COST Action CA17108 and has been partially financed by different specific contracts from the Health and Consumption Service of the València City Council.

REFERENCES

1. Kraemer MUG, Sinka ME, Duda KA, Mylne AQN, Shearer FM, Barker CM *et al. The global distribution of the arbovirus vectors Aedes aegypti and Ae. albopictus*. eLife. 2015 Jun 30;4:e08347. doi: https://dx.doi.org/10.7554/eLife.08347

2. Vazeille M, Moutailler S, Coudrier D, Rousseaux C, Khun H, Huerre M *et al. Two chikungunya isolates from the outbreak of La Reunion (Indian Ocean) exhibit different patterns of infection in the mosquito, Aedes albopictus.* PloS One. 2007 Nov 14;2(11):e1168. doi: https://dx.doi.org/10.1371/journal.pone.0001168

3. Zhao H, Zhang FC, Zhu Q, Wang J, Hong WX, Zhao LZ *et al. Epidemiological and virological characterizations of the 2014 dengue outbreak in Guangzhou, China.* PloS One; 2016 Jun 3;11(6):e0156548. doi: https://dx.doi.org/10.1371/journal.pone.0156548

4. McKenzie BA, Wilson AE, Zohdy S. *Aedes albopictus is a competent vector of Zika virus: A meta-analysis.* PloS One. 2019 May 21;14(5):e0216794. doi: https://dx.doi.org/10.1371/journal.pone.0216794

5. Barzon L, Gobb, F, Capelli G, Montarsi F, Martini S, Riccetti S *et al. Autochthonous dengue outbreak in Italy 2020: clinical, virological and entomological findings.* J Travel Med. 2021 Dec 29;28(8):taab130. doi: https://dx.doi.org/10.1093/jtm/taab130

6. Jourdain F, Roiz D, de Valk H, Noël H, L'ambert G, Franke F *et al. From importation to autochthonous transmission: Drivers of chikungunya and dengue emergence in a temperate area*. PloS Negl Trop Dis. 2020 May 11;14(5):e0008320. doi: https://dx.doi.org/10.1371/journal.pntd.0008320

7. Gjenero-Margan I, Aleraj B, Krajcar D, Lesnikar V, Klobučar A, Pem-Novosel I *et al. Autochthonous dengue fever in Croatia, August-September 2010*. Euro Surveill. 2011 Mar 3;16(9):19805. [consultado 10 agosto 2022]. Disponible en: https://www.eurosurveillance.org/content/10.2807/ese.16.09.19805-en

8. Venturi G, Di Luca M, Fortuna C, Remoli ME, Riccardo F, Severini F, Toma L *et al. Detection of a chikungunya outbreak in central Italy, August to September 2017*. Euro Surveill. 2017 Sep;22(39):17-00646. doi: https://dx.doi.org/10.2807/1560-7917.ES.2017.22.39.17-00646

9. European Center for Disease Control (ECDC). *Local transmission of dengue fever in France and Spain-2018*. Rapid Risk Assessment: 13 pp. [consultado 10 agosto 2022]. Disponible en https://www.ecdc.europa.eu/sites/default/files/documents/08-10-2018-RRA-Dengue-France.pdf

10. Brady OJ, Hay SI. *The first local cases of Zika virus in Europe*. Lancet. 2019 Nov 30;394(10213):1991-1992. doi: https://dx.doi.org/10.1016/S0140-6736(19)32790-4

11. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL *et al. The global distribution and burden of dengue*. Nature. 2013 Apr 25;496(7446):504-7. doi: https://dx.doi.org/10.1038/nature12060

12. European Centre for Disease Prevention and Control (ECDC). *Rapid risk assessment: Autochthonous cases of dengue in Spain and France, 1 October 2019*. [consultado 10 agosto 2022]. Disponible en: https://www.ecdc.europa.eu/sites/portal/files/documents/RRA-dengue-in-Spain-and-France.pdf

13. Aranda C, Martínez MJ, Montalvo T, Eritja R, Navero-Castillejos J, Herreros E *et al. Arbovirus surveillance: first dengue virus detection in local Aedes albopictus mosquitoes in Europe, Catalonia, Spain, 2015*. Euro Surveill. 2018 Nov;23(47):1700837. doi: https://dx.doi.org/10.2807/1560-7917.es.2018.23.47.1700837

14. Baldacchino F, Caputo B, Chandre F, Drago A, della Torre A, Montarsi F *et al. Control methods against invasive Aedes mosquitoes in Europe: a review*. Pest Manag Sci. 2015 Nov;71(11):1471-85. doi: https://dx.doi.org/10.1002/ps.4044

15. Pichler V, Bellini R, Veronesi R, Arnoldi D, Rizzoli A, Lia RP *et al. First evidence of resistance to pyrethroid insecticides in Italian Aedes albopictus populations 26 years after invasion*. Pest Manag Sci. 2018 Jun;74(6):1319-1327. doi: https://dx.doi.org/10.1002/ps.4840

16. Aranda C, Eritja R, Roiz, D. *First record and establishment of the mosquito Aedes albopictus in Spain*. Med Vet Entomol. 2006 Mar;20(1):150-152. doi: https://dx.doi.org/10.1111/j.1365-2915.2006.00605.x

17. Lucientes-Curdi J, Molina-Moreno R, Amela-Heras C, Simon-Soria F, Santos-Sanz S, Sánchez-Gómez A *et al. Dispersion of Aedes albopictus in the Spanish Mediterranean Area*. Eur J Public Health. 2014 Aug;24(4):637-640. doi: https://dx.doi.org/10.1093/eurpub/cku002

18. Ministerio de Sanidad, Consumo y Bienestar Social (MSCBS). 2019. *Vigilancia Entomológica: resultados de 2018*. [consultado 10 agosto 2022]. Disponible en: https://www.mscbs.gob.es/ciudadanos/saludAmbLaboral/docs/Encuesta_Vigilancia_Entomologica.2018.pdf

19. Melero-Alcibar R, Tello A, Marino E, Vázquez, MA. *Aedes (Stegomyia) albopictus (Skuse, 1894) (Diptera, Culicidae) first detection for the Community of Madrid, Spain*. Boln. Asoc. Esp. Ent. 2017; 41 (3-4): 515-519.

20. Ordóñez Iriarte JM, Iriso Calle A, Fuster Lorán F, Tello Fierro A, Junco Bonet A, De la Cruz Pérez M. *Vigilancia entomológica de Aedes (Stegomyia) albopictus (Skuse, 1894) en la Comunidad de Madrid: avance de resultados 2016-2020*. Rev. Salud ambient. 2021; 21(2):160-169.

21. Bueno-Marí R, Quero de Lera F. *Vigilancia entomológica frente a mosquitos invasores en la ciudad de Valencia: primer registro del mosquito tigre, Aedes albopictus (Skuse, 1894), en el municipio*. Zool Baetica. 2015; 26, 145-151.

22. Bueno-Marí R, Quero de Lera F. *Gestión vectorial de los casos de arbovirosis notificados en la ciudad de Valencia, España (2016-2018)*. 2021 May 10;95:e202105064.

23. Tancredi A, Papandrea D, Marconcini M, Carballar-Lejarazu R, Casas-Martinez M, Lo E *et al. Tracing temporal and geographic distribution of resistance to pyrethroids in the arboviral vector Aedes albopictus*. PLoS Negl Trop Dis. 2020 Jun 22;14(6):e0008350. doi: https://dx.doi.org/10.1371/journal.pntd.0008350

24. Huang Y-JS, Higgs S, Vanlandingham DL. *Biological control strategies for mosquito vectors of arboviruses*. Insects. 2017 Feb 10;8(1):21. doi: https://dx.doi.org/10.3390/insects8010021

25. Alphey N Bonsall, MB. *Genetics-based methods for agricultural insect pest management*. Agric For Entomol. 2018 May;20(2):131-140. doi: https://dx.doi.org/10.1111/afe.12241

26. Sicard M, Bonneau M, Weill M. *Wolbachia prevalence, diversity, and ability to induce cytoplasmic incompatibility in mosquitoes*. Curr Opin Insect Sci. 2019 Aug;34:12-20. doi: https://dx.doi.org/10.1016/j.cois.2019.02.005

27. Achee NL, Grieco JP, Vatandoost H, Seixas G, Pinto J, Ching-Ng *et al. Alternative strategies for mosquito-borne arbovirus control*. PLoS Negl Trop Dis. 2019 Jan 3;13(1):e0006822. doi: https://dx.doi.org/10.1371/journal.pntd.0006822

28. Landmann F. *The Wolbachia endosymbionts*. Microbiol Spectr. 2019 Mar;7(2). doi: https://dx.doi.org/10.1128/microbiolspec.BAI-0018-2019

29. Olanratmanee P, Baimai V, Ahantarig A. Trinachartvanit W. *Novel supergroup U Wolbachia in bat mites of Thailand*. Southeast Asian J Trop Med Public Health 2021 Feb;52:48-55.

30. Werren JH, Baldo L, Clark ME. *Wolbachia: master manipulators of invertebrate biology*. Nat Rev Microbiol. 2008 Oct;6(10):741-51. doi: https://dx.doi.org/10.1038/nrmicro1969

31. Hoffmann AA, Ross PA, Rašić G. *Wolbachia strains for disease control: ecological and evolutionary considerations*. Evol Appl. 2015 Sep;8(8):751-68. doi: https://dx.doi.org/10.1111/eva.12286

32. Yen PS, Failloux AB. *A Review: Wolbachia-based population replacement for mosquito control shares common points with genetically modified control approaches*. Pathogens. 2020 May 22;9(5):404. doi: https://dx.doi.org/10.3390/pathogens9050404

33. Armbruster P, Damsky WE, Giordano R, Birungi J, Munstermann L, Conn J. *Infection of New- and Old-World*

Aedes albopictus (Diptera: Culicidae) by the intracellular parasite Wolbachia: Implications for host mitochondrial DNA evolution. J Med Entomol. 2003 May;40(3):356-360. doi: https://dx.doi.org/10.1603/0022-2585-40.3.356

34. Bourtzis K, Dobson SL, Xi Z, Rasgon JL, Calvitti M, Moreira LA *et al. Harnessing mosquito-Wolbachia symbiosis for vector and disease control*. Acta Trop. 2014 Apr;132 Suppl:S150-163. doi: https://dx.doi.org/10.1016/j.actatropica.2013.11.004

35. Zhou W, Rousset F, O'Neill S. *Phylogeny and PCR-based classification of Wolbachia strains using wsp gene sequences*. Proc Biol Sci. 1998 Mar 22;265(1395):509-515. doi: https://dx.doi.org/10.1098/rspb.1998.0324

36. Kaur R, Shropshire JD, Cross, KL, Leigh B, Mansueto AJ, Stewart V *et al. Living in the endosymbiotic world of Wolbachia: A centennial review*. Cell Host Microbe. 2021 Jun 9;29(6):879-893. doi: https://dx.doi.org/10.1016/j.chom.2021.03.006

37. Calvitti M, Moretti R, Lampazzi E, Bellini R, Dobson SL. *Characterization of a new Aedes albopictus (Diptera: Culicidae)-Wolbachia pipientis (Rickettsiales: Rickettsiaceae) symbiotic association generated by artificial transfer of the wPip strain from Culex pipiens (Diptera: Culicidae)*. J Med Entomol. 2010 Mar;47(2):179-187. doi: https://dx.doi.org/10.1603/me09140

38. Caputo B, Moretti R, Manica M, Serini P, Lampazzi E, Bonanni M *et al. 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. 2020 Apr;76(4):1324-1332. doi: https://dx.doi.org/10.1002/ps.5643

39. Ahmad NA, Vythilingam I, Lim YAL, Zabari NZAM, Lee HL. *Detection of Wolbachia in Aedes albopictus and their effects on chikungunya virus*. Am J Trop Med Hyg. 2017 Jan 11;96(1):148-156. doi: https://dx.doi.org/10.4269/ajtmh.16-0516

40. Schaffner F, Angel G, Geoffroy B, Hervy JO, Rhaeim A. *The mosquitoes of Europe/Les moustiques d'Europe. An identification and training programme/Logiciel d'identification et d'enseignement*. Didactiques. Montpellier:IRD Editions & EID Méditerranée. 2001.

41. Carvajal TM, Hashimoto K, Harnandika RK, Amalin DM, Watanabe K. *Detection of Wolbachia in field-collected Aedes aegypti mosquitoes in metropolitan Manila, Philippines*. Parasit Vectors. 2019 Jul 24;12(1):361. doi: https://dx.doi.org/10.1186/s13071-019-3629-y

42. Wu Y, Zheng X, Zhang M, He A, Li Z, Zhan X. *Cloning and functional expression of Rh50-like glycoprotein, a putative ammonia channel, in Aedes albopictus mosquitoes*. J Insect Physiol. 2010 Nov;56(11):1599-610. doi: https://dx.doi.org/10.1016/j.jinsphys.2010.05.021

43. Staden R, Beal KF, Bonfield JK. *The Staden package, 1998*. Methods Mol Biol. 2000;132:115-30. doi: https://dx.doi.org/10.1385/1-59259-192-2:115

44. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. *Basic local alignment search tool*. J Mol Biol. 1990 Oct 5;215(3):403-410. doi: https://dx.doi.org/10.1016/S0022-2836(05)80360-2

45. Tamura K, Stecher G, Kumar S. *MEGA11: Molecular Evolutionary Genetics Analysis Version 11*. Mol Biol Evol. 2021 Jun 25;38(7):3022-3027. doi: https://dx.doi.org/10.1093/molbev/msab120

46. Tsai KH, Lien JC, Huang CG, Wu WJ, Chen WJ. *Molecular (Sub)grouping of endosymbiont Wolbachia Infection among mosquitoes of Taiwan*. J Med Entomol. 2004 Jul;41(4):677-683. doi: https://dx.doi.org/10.1603/0022-2585-41.4.677

47. Mavingui P, Moro CV, Tran-Van V, Wisniewski-Dyé F, Raquin V, Minard G *et al. Whole-genome sequence of Wolbachia strain wAlbB, an endosymbiont of tiger mosquito vector Aedes albopictus*. J Bacteriol. 2012 Apr;194(7):1840. doi: https://dx.doi.org/10.1128/JB.00036-12

48. Braig HR, Zhou W, Dobson SL, O'Neill SL. *Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont Wolbachia pipientis*. J Bacteriol. 1998 May;180(9):2373-2378. doi: https://dx.doi.org/10.1128/jb.180.9.2373-2378.1998

49. Noda H, Miyoshi T, Zhang Q, Watanabe K, Deng K,

Hoshizaki S. *Wolbachia infection shared among planthoppers (Homoptera: Delphacidae) and their endoparasite (Strepsiptera: Elenchidae): a probable case of interspecies transmission.* Mol Ecol. 2001 Aug;10(8):2101-2106. doi: https://dx.doi.org/10.1046/j.0962-1083.2001.01334.Xx

50. Kittayapong P, Jamnongluk W, Thipaksorn A, Milne JR, Sindhusake C. *Wolbachia infection complexity among insects in the tropical rice-field community. Wolbachia infection complexity among insects in the tropical rice-field community.* doi: https://dx.doi.org/10.1046/j.1365-294X.2003.01793.x

51. Paupy C, Delatte H, Bagny L, Corbel V, Fontenille D. *Aedes albopictus, an arbovirus vector: from the darkness to the light.* Microbes Infect. 2009 Dec;11(14-15):1177-1185. doi: https://dx.doi.org/10.1016/j.micinf.2009.05.005

52. Calvitti M, Moretti R, Skidmore AR, Dobson SL. *Wolbachia strain wPip yields a pattern of cytoplasmic incompatibility enhancing a Wolbachia-based suppression strategy against the disease vector Aedes albopictus.* Parasit Vectors. 2012 Nov 12;5:254. doi: https://dx.doi.org/10.1186/1756-3305-5-254

53. Moretti R, Calvitti M. *Male mating performance and cytoplasmic incompatibility in a wPip Wolbachia trans-infected line of Aedes albopictus (Stegomyia albopicta).* Med Vet Entomol. 2013 Dec;27(4):377-386. doi: https://dx.doi.org/10.1111/j.1365-2915.2012.01061.x

54. Tortosa P, Charlat S, Labbé P, Dehecq JS, Barré H, Weill M. *Wolbachia age-sex-specific density in Aedes albopictus: A host evolutionary response to cytoplasmic incompatibility?* PLoS One. 2010 Mar 16;5(3):e9700. doi: https://dx.doi.org/10.1371/journal.pone.0009700

55. Hu Y, Xi Z, Liu X, Wang J, Guo Y, Ren D *et al. Identification and molecular characterization of Wolbachia strains in natural populations of Aedes albopictus in China.* Parasit Vectors. 2020 Jan 14;13(1):28. doi: https://dx.doi.org/10.1186/S13071-020-3899-4

56. Zhang D, Zhan X, Wu X, Yang X, Liang G, Zheng Z *et al. A field survey for Wolbchia and phage WO infections of Aedes albopictus in Guangzhou City, China.* Parasitol Res. 2014 Jan;113(1):399-404. doi: https://dx.doi.org/10.1007/S00436-013-3668-9

57. Kittayapong P, Baimai V, O'Neill SL. *Field prevalence of Wolbachia in the mosquito vector Aedes albopictus.* Am J Trop Med Hyg. 2002 Jan;66(1):108-111. doi: https://dx.doi.org/10.4269/ajtmh.2002.66.108

58. Park CH, Lim HW, Kim HW, Lee WG, Roh JY, Park MY, Shin EH. *High prevalence of Wolbachia infection in Korean populations of Aedes albopictus (Diptera: Culicidae).* J. Asia. Pac. Entomol. 2016 March; 19(1):191-194. doi: https://dx.doi.org/10.1016/j.aspen.2015.12.014

59. Anderson M, Rustin R, Eremeeva M. *Pilot survey of mosquitoes (Diptera: Culicidae) from southeastern Georgia, USA for Wolbachia and Rickettsia felis (Rickettsiales: Rickettsiaceae).* Vector Borne Dis. 2019 Apr-Jun;56(2):92-97. doi: https://dx.doi.org/10.4103/0972-9062.263714

60. De Albuquerque AL, Magalhães T, Ayres CFJ. *High prevalence and lack of diversity of Wolbachia pipientis in Aedes albopictus populations from Northeast Brazil.* Mem Inst Oswaldo Cruz. 2011 Sep;106(6):773-776. doi: https://dx.doi.org/10.1590/S0074-02762011000600021

61. Torres-Monzón JA, Casas-Martínez M, López-Ordóñez T. *Infection of Aedes mosquitoes by native Wolbachia in urban cemeteries of Southern Mexico.* Salud Publica Mex. 2020 Jul-Aug;62(4):447-449. doi: https://dx.doi.org/10.21149/10163

62. Minard G, Tran FH, Van VT, Goubert C, Bellet C, Lambert G *et al. French invasive Asian tiger mosquito populations harbor reduced bacterial microbiota and genetic diversity compared to Vietnamese autochthonous relatives.* Front Microbiol. 2015 Sep 22;6:970. doi: https://dx.doi.org/10.3389/fmicb.2015.00970

63. Maimusa HA, Ahmad AH, Kassim NFA, Rahim J. *Age-Stage, Two-sex life table characteristics of Aedes albopictus and Aedes Aegypti in Penang Island, Malaysia.* J Am Mosq Control Assoc. 2016 Mar;32(1):1-11. doi: https://dx.doi.org/10.2987/moco-32-01-1-11.1

64. Calvitti M, Moretti R, Porretta D, Bellini R, Urbanelli S. *Effects on male fitness of removing Wolbachia infections from the mosquito Aedes albopictus.* Med Vet Entomol. 2009 Jun;23(2):132-140. doi: https://dx.doi.org/10.1111/J.1365-2915.2008.00791.x

65. *Regulation (EU) Nº 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products.* [consultado 1 junio 2022]. Disponible en: *https://eur-lex.europa.eu/legal-content/en/TXT/?uri=-CELEX%3A32012R0528*

66. *Commission Implementing Decision (EU) 2018/1623 of 29 October 2018 pursuant to Article 3(3) of Regulation (EU) Nº 528/2012 of the European Parliament and of the Council on mosquitoes non-naturally infected with Wolbachia used for vector control purposes*. [consultado 1 junio 2022]. Disponible en: *https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv%3AOJ.L_.2018.271.01.0030.01.ENG&toc=OJ%3AL%3A2018%3A271%3ATOC*

Annex I

Demographic profile (sex, site code, location), *Wolbachia* detection status and of supergroup classification for all individual adult *Aedes albopictus* mosquitoes used in this study.

Sample	Sex	Site	Coordinates		*w*AlbA	*w*AlbB	Infection
			Latitude	*Longitude*			
ALB1W0L1F	F	D1	39,4727	-0.3695	+	+	AB
ALB1W0L1M	M	D1	39,4727	-0.3695	+	+	AB
ALB2W0L1F	F	D2	39,4589	-0.3759	+	+	AB
ALB2W0L1M	M	D2	39,4589	-0.3759	+	-	A
ALB3W0L1F	F	D3	39,4769	-0.3867	+	+	AB
ALB3W0L1M	M	D3	39,4769	-0.3867	-	+	B
ALB3W0L2M	M	D3	39,4769	-0.3867	-	+	B
ALB4W0L1F	F	D4	39,4916	-0.4036	+	+	AB
ALB4W0L1M	M	D4	39,4916	-0.4036	+	+	AB
ALB5W0L1F	F	D5	39,4869	-0.382	+	-	A
ALB5W0L1M	M	D5	39,4869	-0.382	+	+	AB
ALB5W0L2M	M	D5	39,4869	-0.382	-	+	B
ALB6W0L1F	F	D6	39,4776	-0.3641	+	-	A
ALB6W0L1M	M	D6	39,4776	-0.3641	-	+	B
ALB7W0L1F	F	D7	39,4667	-0.3958	+	+	AB
ALB7W0L2F	F	D7	39,4667	-0.3958	-	-	0
ALB7W0L1M	M	D7	39,4667	-0.3958	+	+	AB
ALB8W0L1F	F	D8	39,4531	-0.4043	+	-	A
ALB8W0L2F	F	D8	39,4531	-0.4043	+	+	AB
ALB8W0L1M	M	D8	39,4531	-0.4043	-	-	0
ALB9W0L1F	F	D9	39,4446	-0.3944	+	+	AB
ALB9W0L1M	M	D9	39,4446	-0.3944	+	+	AB
ALB9W0L2M	M	D9	39,4446	-0.3944	+	+	AB
ALB10W0L1F	F	D10	39,4444	-0.3661	+	+	AB
ALB10W0L2F	F	D10	39,4444	-0.3661	+	+	AB

Annex I (continues)
Demographic profile (sex, site code, location), *Wolbachia* detection status and of supergroup classification for all individual adult *Aedes albopictus* mosquitoes used in this study.

Sample	Sex	Site	Coordinates		*w*AlbA	*w*AlbB	Infection
			Latitude	*Longitude*			
ALB10W0L1M	M	D10	39,4444	-0.3661	-	+	B
ALB11W0L1F	F	D11	39,4666	-0.3267	+	+	AB
ALB11W0L1M	M	D11	39,4666	-0.3267	-	+	B
ALB12W0L1F	F	D12	39,4673	-0.34	+	+	AB
ALB12W0L1M	M	D12	39,4673	-0.34	-	+	B
ALB13W0L1F	F	D13	39,4733	-0.3425	+	+	AB
ALB13W0L1M	M	D13	39,4733	-0.3425	+	+	AB
ALB14W0L1F	F	D14	39,4816	-0.358	+	+	AB
ALB14W0L1M	M	D14	39,4816	-0.358	+	+	AB
ALB15W0L1F	F	D15	39,493	-0.3815	+	+	AB
ALB15W0L1M	M	D15	39,493	-0.3815	+	+	AB
ALB16W0L1F	F	D16	39,4984	-0.3962	+	+	AB
ALB16W0L2F	F	D16	39,4984	-0.3962	+	+	AB
ALB16W0L3F	F	D16	39,4984	-0.3962	+	+	AB
ALB16W0L4F	F	D16	39,4984	-0.3962	+	+	AB
ALB16W0L1M	M	D16	39,4984	-0.3962	+	+	AB
ALB16W0L2M	M	D16	39,4984	-0.3962	+	+	AB
ALB16W0L3M	M	D16	39,4984	-0.3962	-	-	0
ALB16W0L4M	M	D16	39,4984	-0.3962	+	+	AB
ALB17W0L1F	F	D17	39,5229	-0.3784	+	+	AB
ALB17W0L1M	M	D17	39,5229	-0.3784	+	+	AB
ALB18W0L1F	F	D18	39,5018	-0.4241	+	+	AB
ALB18W0L1M	M	D18	39,5018	-0.4241	-	+	B
ALB19W0L1F	F	D19	39,4222	-0.3396	+	+	AB
ALB19W0L1M	M	D19	39,4222	-0.3396	-	+	B