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Wolbachia infection prevalence as common insects' endosymbiont in the rural area of Yogyakarta, Indonesia

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Abstract. *Kumalawati DA, Supriyati E, Rachman MP. Oktriani R, Kurniasari I, Candrasari DS, Hidayati L, Handayaningsih AE, Probowati VC, Arianto B, Wardana DS, Pramuko NB, Utari A, Tantowijoyo W, Arguni E. 2020.* Wolbachia *infection prevalence as common insects' endosymbiont in the rural area of Yogyakarta, Indonesia*. *Biodiversitas 21: 5608-5614.* Control for mosquito-borne diseases such as dengue and chikungunya using vectors is urgently required. The World Mosquito Program, a multinational collaborative research program, is currently studying for the ability of *Wolbachia*-infected *Aedes aegypti* to control dengue. Community concerns on *Wolbachia*'s natural existence in their surrounding living area. This study presents the field study of *Wolbachia pipientis* in insects commonly found in rural daily life. *W. pipientis* is an endosymbiotic bacterium commonly found in arthropods. Insects were collected from five villages in Sleman and Bantul District, Yogyakarta Province, Indonesia, from July to December 2012 and screened for *Wolbachia* infection using PCR. One hundred insects, including butterflies, moths, mosquitoes, flies, were collected. The results indicated that 44.9% of identified insect species were positive for *Wolbachia pipientis*, which support the existing data from other regions on the spread of *Wolbachia* infection in insects.

Keywords: Arthropods, bacteria, endosymbiotic, insects, *Wolbachia pipientis*

INTRODUCTION

Dengue infection is a viral disease estimated to cause a high public health burden in the world (Bhatt et al. 2013; Indriani et al. 2018). This infection is primarily transmitted by *Aedes aegypti*. The technology proposed by the World Mosquito Program Yogyakarta in 2012 is to use artificially infected *Wolbachia* bacteria to *Aedes aegypti* as biological dengue control in open field trials in Yogyakarta, Indonesia as a collaborator with Australia, Vietnam, and Brazil. Yogyakarta Province is one of the ten most prevalent provinces of dengue cases reported yearly (Kementrian Kesehatan RI 2010). Since community acceptability is very crucial to this trial, one of the community engagement strategies was to address their concerns about this new technology. One of the initial problems that emerged from the research site community was: what is Wolbachia? Is it a foreign biological creature or one commonly found in our daily lives? (WMP 2015, data not shown). The aim of this study was to respond to the concerns of the community.

Wolbachia pipientis is currently a large and diverse bacterial species within the *Alpha-proteobacteria.* They are most closely related to Rickettsia, Anaplasma, and Ehrlichia. However, unlike their relatives, *Wolbachia* only infects arthropods and some nematodes and has never been found in invertebrates. *Wolbachia s*trains in supergroups A & B only infect insects. They are very distantly related to those infecting nematodes, which belong to supergroups C & D and are currently being considered for reclassification into separate species (Pfarr et al. 2007). *Wolbachia*

pipientis was initially discovered in 1924 in the ovaries of the mosquito *Culex pipiens*. In the initial discovery, it was screened as a potential new human pathogen. However, tests involving its introduction into mice showed it is non-pathogenic and a common symbiont of mosquitoes. *Wolbachia* does not produce spores nor contain plasmids. There are several different types (strains) of *Wolbachia*, usually associated with a single host species (Werren et al. 2008). In recent decades, screening of insect species using PCR amplification and DNA sequencing has revealed that *Wolbachia* is estimated to infect between 20-76% of insect species worldwide (Jeyaprakash and Hoy 2000; Stevens et al. 2001; Weinert et al. 2007; Werren and Windsor 2000; Werren et al. 1995). It suggests that a more likely figure of 75% of all arthropod species are infected. They do not infect humans or any other vertebrates (Bandi et al. 2001; Bouchon et al. 1998; Breeuwer and Jacobs 1996; Rowley et al. 2004; Taylor and Hoerauf 1999; Woo Oh et al. 2000). *Aedes aegypti* does not naturally harbor *Wolbachia* (Ruang-Areerate and Kittayapong 2006), although many other species of mosquito are known to be infected (Rasgon and Scott 2004; Tsai et al. 2004).

Wolbachia strains are typically 0.5-1 µm in size and live as highly specialized obligate endosymbionts in the cytoplasm of the host cells. *Wolbachia* strains are neither infectious nor can be transmitted from insect to insect as a pathogen. Instead, they are passed from parent to offspring through the insect eggs. Artificial transfer across different species is complicated and has only been obtained in the laboratory following microinjection of purified *Wolbachia* into the insects. Studies in Indonesia found that *Wolbachia* infects butterflies, moths, mosquitoes, and ants (Lohman et al. 2008; Narita et al. 2007; Wenseleers et al. 1998).

Yogyakarta Province is located in the south-central of Java Island (Figure 1), is one of the most populous provinces in Indonesia, consisting of five districts. The total population of Sleman District is 1,128,943, with an area of 574.82 square kilometers. The total population of Bantul District is 934,674; with an area of 506.85 square kilometers. The results of the study could support existing data that *Wolbachia* is common bacteria that naturally infect common insects around human settlements in Yogyakarta.

MATERIALS AND METHODS

Sampling location and time

The research was conducted in five study sites in Yogyakarta Province, Indonesia, i.e.: 2 villages in Bantul District (Jomblangan and Singosaren); and three villages in Sleman District (Kalitirto, Nogotirto, and Kronggahan) (Figure 1). Samples were collected from July to December 2012.

Sample collection

We used purposive random sampling consisting of three collection methods, which are direct catch (using bananas as bait), sweep-net, and light trap for nocturnal insects. The samples were indoor, outdoor, and agricultural insects. Samples were sent to taxonomists at the Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia for species identification. One to five samples per species were collected for diagnostic assessment. The samples were stored in 70% ethanol and kept under -20 ºC for further analysis.

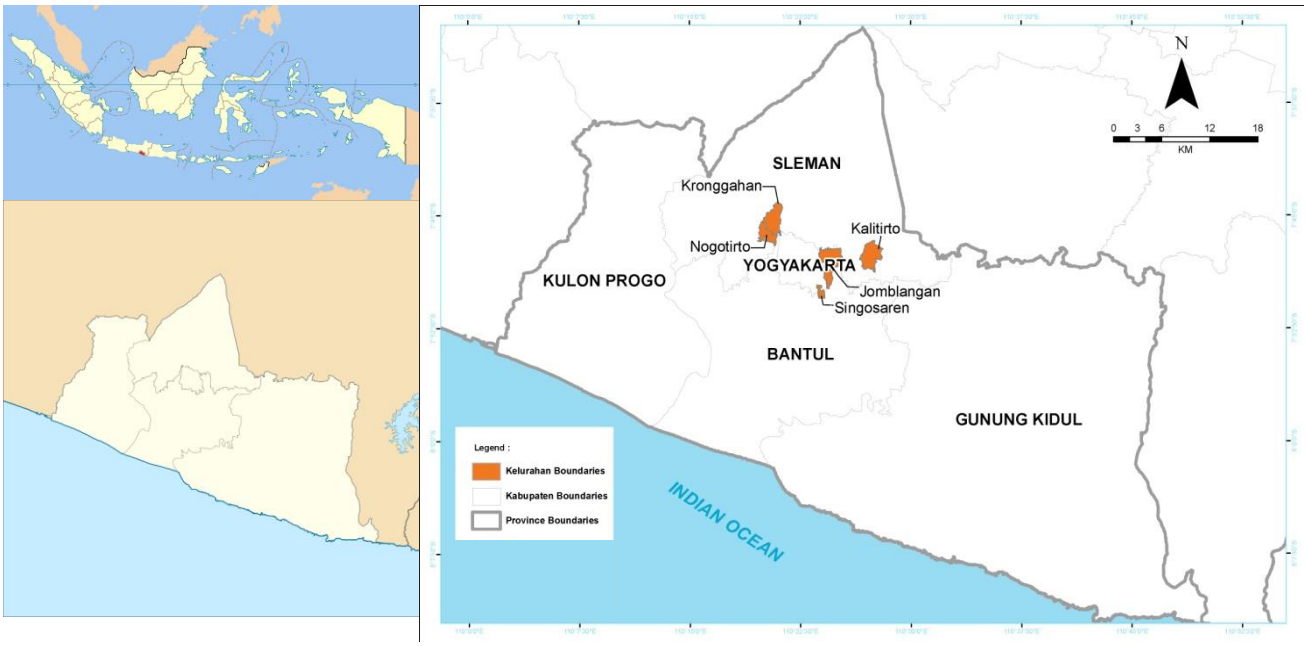

Figure 1. Research site in Yogyakarta Province, Indonesia (*left*) and sampling areas (*right*)

Wolbachia detection by polymerase chain reaction (PCR) and data analysis

The thorax and abdomen from each individual (or the whole body for small insects) were taken for the extraction process. DNA extraction was performed using a commercial DNA isolation kit (Genejet Genomic DNA Purification Kit, Thermo Scientific). wMel+ *Aedes aegypti* was used as a positive control assay. The extraction procedure followed the Thermo Scientific Company's procedure with modification. The quality of genomic DNA extracted from samples was checked by PCR targeting butterfly DNA using arthropod-specific 28S rRNA primers, amplified as previously described (Werren et al. 1995).

The PCR to detect *Wolbachia* was performed by Maxima Hot Start Green PCR Master Mix (2X) (Thermo Scientific) using *Wolbachia* general primers against the *Wolbachia* surface protein gene (*wsp* gene) (Braig et al., 1998). These primers generate a 610bp product. The primers were diluted to obtain the appropriate and optimum concentration for the PCR procedure, which was 10µM for *wsp* primers.

Primer 81F: 5'-TGGTCCAATAAGTGATGAAGAAAC-3'
Primer 691R: 5'-AAAAATTAAACGCTACTCCA-3'

The total volume of the PCR mix was 20 µL. PCR reactions were run at 95°C for 3 min followed by 35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min using a thermal cycler (BioRad C-1000 Touch). Horizontal agarose gel electrophoresis apparatus (Mupid® Ex-U) was used to perform DNA analysis. Four uL of 6x loading dye was added to the PCR tube and run in a 2% agarose gel containing GelRed Nucleic Acid Stain. The PCR product should be about 610bp in size compared to the DNA Ladder (100bp Ladder Thermo Scientific). The band was visualized using Geldoc Documentation System (Protein Simple). The 610 bp band was used to determine the presence of *Wolbachia*. The percentage of *Wolbachia* in insects was counted with the following formula :

$$\frac{\text{Total number of insects species positive for } \textit{Wolbachia}}{\text{Total number of identified insects species}} \times 100\%$$

Ethics approval

This study was part of the World Mosquito Program (WMP) Yogyakarta (formerly known as the Eliminate Dengue Program) Phase 1. Ethical approval was obtained from the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health and Nursing Universitas Gadjah Mada, Indonesia (approval number KE/FK/01/EC/2012)

RESULTS AND DISCUSSIONS

Results

From 100 collected insects, 49 species were identified from 8 different orders. Those were screened for *Wolbachia* infection, of which 44.9% were positive for the bacteria (Table 1). *Wolbachia* were found in each of the major orders, including Diptera, Coleoptera, and Lepidoptera, Hymenoptera, and Orthoptera. The order Lepidoptera has the highest distribution of *Wolbachia* (12 of 25 individuals). Figure 2 shows amplification of 610 bp DNA fragment by using 81F and 691R primers for *wsp* gene (figure represented examples of positive and negative samples of analyzed insects)

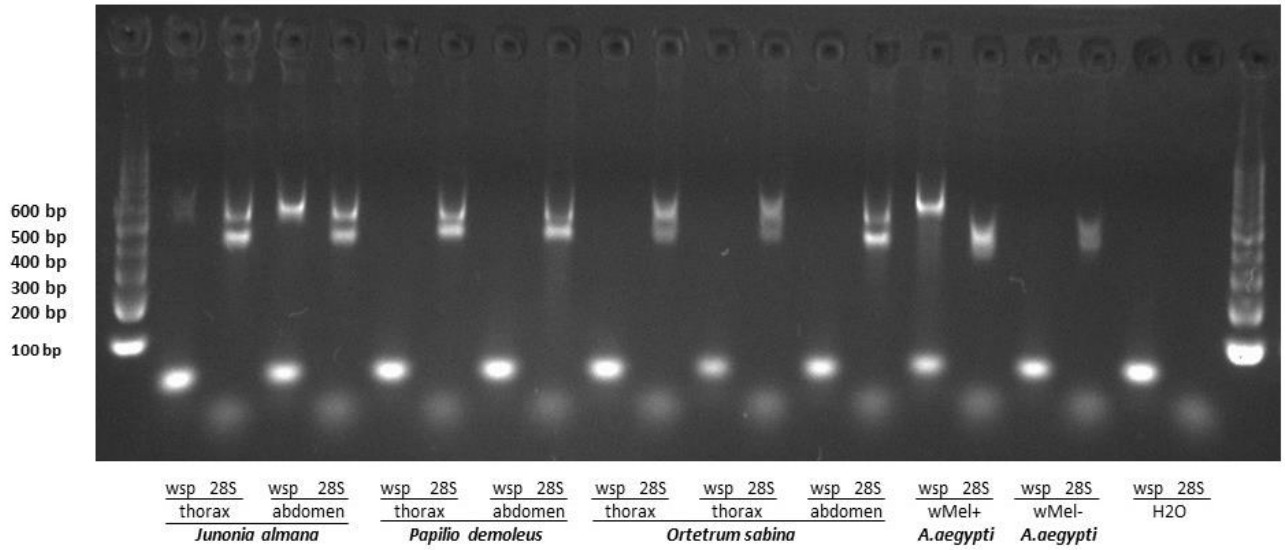

Figure 2. Amplification result of *wsp* primers set in *Junonia almana*, but not in *Papilio demoleus* and *Orthetrum sabina*. An amplification band of 28S rRNA was detected in all samples. wMel+ *Aedes aegypti* was a positive control, wMel- *Aedes aegypti* and H_2O were negative control.

Table 1. The presence of *Wolbachia* in collected insect samples (T: Thorax; A: Abdomen; WB: Whole Body)

Order	Common name	Species	Body part used for DNA isolation	Presence of *Wolbachia*
Blattodea	Cockroach	*Periplaneta* spp.	T, A	Negative
Coleoptera	Ladybug	*Henosepilachna sparsa*	WB	Positive
	Beetle	*Aspidomorph amiliaris*	WB	Negative
	Beetle	*Apoderus* sp.	WB	Positive
	Beetle	*Coelophora reniplagiata*	WB	Negative
	Beetle	*Paederus* sp.	WB	Negative
Diptera	Flies	*Drosophila melanogaster*	WB	Positive
	Mosquitoes	*Aedes albopictus*	WB	Positive
	Mosquitoes	*Aedes aegypti*	WB	Negative
Hemiptera	Ladybug	*Leptocorisa acuta*	WB	Negative
Hymenoptera	Bees	*Apis mellifera*	T, A	Positive
	Wasp	*Poliste sagitarius*	T, A	Negative
	Wasp	*Delta camponiforme*	T, A	Positive
	Wasp	*Sceliphron* sp.	T, A	Positive
	Wasp	*Vespa analis*	T, A	Negative
	Wasp	*Xylocopa confusa*	T, A	Negative
Lepidoptera	Butterfly	*Papilio memnon*	T, A	Positive
	Butterfly	*Catopsilia pomona*	T, A	Positive
	Butterfly	*Neptis hylas*	WB	Positive
	Butterfly	*Mycalesis horsfieldi*	T, A	Positive
	Butterfly	*Eurema blanda*	T, A	Negative
	Butterfly	*Eurema candida*	T, A	Negative
	Butterfly	*Elymnia hypermnestra*	T, A	Positive
	Butterfly	*Elymnias nesaea*	T, A	Negative
	Butterfly	*Papilio polytes*	T, A	Positive
	Butterfly	*Delias hyparete*	T, A	Negative
	Butterfly	*Melanitis leda*	T, A	Positive
	Butterfly	*Junonia atlites*	T, A	Positive
	Butterfly	*Junonia almana*	T, A	Positive
	Butterfly	*Papilio demoleus*	T, A	Negative
	Butterfly	*Hypolimnas bolina M*	T, A	Positive
	Butterfly	*Doleschalia bisaltide*	T, A	Negative
	Butterfly	*Junonia hedonia*	T, A	Negative
	Butterfly	*Euploea midamus*	T, A	Negative
	Butterfly	*Appias olferna*	T, A	Negative
	Butterfly	*Mycalesis* sp.	T, A	Positive
	Butterfly	*Acraea terpsicore*	T, A	Negative
	Butterfly	*Graphium agamemnon*	T, A	Positive
	Butterfly	*Eurema hecabe*	T, A	Negative
Odonata	Dragonfly	*Orthetrum sabina*	T, A	Negative
	Dragonfly	*Crocothemis servilia*	T, A	Negative
Orthoptera	Grasshopper	*Tenodera fasciata*	T, A	Negative
	Grasshopper	*Oxya intricata*	T, A	Negative
	Grasshopper	*Catantops humilis*	T, A	Negative
	Grasshopper	*Xiphidion maculatum*	T, A	Negative
	Grasshopper	*Euscyrtusconcinnus*	T, A	Positive
	Grasshopper	*Tagaeta marginella*	T, A	Positive
	Grasshopper	*Atractomorpha crenaticeps*	T, A	Positive
	Grasshopper	*Atractomorpha psittacina*	T, A	Negative
Total		49		Pos: 22 Neg: 27

Note: Percentage of *Wolbachia* sp.: 22 / 49 x 100% = 44.9%

Discussion

Wolbachia can spread rapidly among host populations through vertical lineages (vertical transmission from parent to offspring). The presence of *Wolbachia* has an impact on host life and ecology. These include reproductive manipulation. Reproductive manipulations include feminization (male acquires female traits); induction of parthenogenesis (female offspring are produced without fertilization), male embryo killing, and cytoplasmic incompatibility (CI), where sperm from infected males cannot produce viable offspring with females that do not harbor the same bacterial strain (Werren et al. 2008). Therefore, the four strategies mentioned above could enhance the survival and spread of the bacteria in insect populations as the host, even at the expense of host fitness.

From the 49 samples of insects, fragments were appeared in 22 samples using 81 F and 691 R primers for amplification. The 610 bp fragments were almost similar for all positive samples. It indicates the presence of *Wolbachia* in the sample. The 81 F and 691 R primers were targeting the *wsp*. *Wolbachia* surface protein (*WSP*) contains the transmembrane domain and standard signal peptide for secretion. It was one of the most abundantly expressed proteins in the arthropod endosymbiont and shows homologies to the major outer membrane proteins of *Ehrlichia* spp. and related genera. It proves that *wsp* gene can be very useful for phylogenetic studies of arthropod *Wolbachia* because it expresses *WSP* as a membrane protein of the bacterial outer envelope (Bazzochi et al. 2000). For internal control, a primer which amplifies 28S fragment were also included. 28S ribosomal RNA is the structural ribosomal RNA (rRNA) for the large component of eukaryotic cytoplasmic ribosomes, and thus one of the basic components of all eukaryotic cells. The fragment from the positive samples revealed similar results to the fragments from wMel+ mosquito for positive controls. It indicates that *Wolbachia* in wMel+ mosquito has similarity, based on 81 F and 691 R primers amplification, to the *Wolbachia* in most of the wild insects in this study.

Twelve out of 23 butterfly species (Lepidoptera) were positive for *Wolbachia*. The presence of *Wolbachia* in butterfly species was also reported from Western Ghats, India. Twenty-nine species representing five families (Papilionidae, Nymphalidae, Pieridae, Lycaenidae, Hesperiidae) were positive for *Wolbachia* (Salunke et al. 2012). The family Papilionidae, represented by *Papilio demoleus*, showed different results to this study. *P. demoleus* from the Western Ghats were positive for *Wolbachia*, but the Yogyakarta sample showed a negative result. Other species such as *Hypolimnas bolina M* and *Neptis hylas* showed a similar result. In this study, some species from Hymenoptera, including bees and wasps, are positive for *Wolbachia*. *Hyposoter horticola*, a parasitoid of the Glanville fritillary butterfly in the Åland Islands in Finland, was also infected by three *Wolbachia pipientis* strains, i.e. wHho, wHho2, and wHho3 (Duplouy et al. 2015). *Wolbachia* was also reported in *Diprionpini* (L.) and *Neodiprion sertifer* (Hymenoptera) from Northern Italy. The presence of *Wolbachia* in wasps can be diverse among species. Maternal vertical inheritance is considered the primary route of *Wolbachia* transmission from one host generation to another. Another mechanism, horizontal transmission, has been reported in wasps of the genus *Trichogramma*, and the parasitoid *Nasonia* wasp, which allows *Wolbachia* to infect several host species and contributes to its vast host range. The horizontal transmission allows *Wolbachia* to infect several host species and contributes to its immense host range (Pistone et al. 2014).

In this study, 2 out of 5 species of the order Coleoptera were positive for *Wolbachia*. A study by Sontowski et al. (2015) revealed the presence of *Wolbachia* in seven families of Coleoptera (Buprestidae, Hydraenidae, Dytiscidae, Hydrophilidae, Gyrinidae, Haliplidae, and Noteridae) from Europe with an infection rate of 31% in total. The infection rates for each family was ranging from 14% to 63% for families with more than 12 sampled species. There has not been much study on screening for *Wolbachia* infection in beetles (Clark et al. 2001; Weinert et al. 2007), indicating that *Wolbachia* infections in beetles are generally lower compared to other insects orders. The negative result of *Wolbachia* infection also appeared in three species of the order Odonata. This result is different from the study by Salunkhe et al. (2015) that found fifteen species of Odonate representing five families from Central India are positive for *Wolbachia* with an infection rate of 70% (Salunkhe et al. 2015). The prevalence of *Wolbachia* infection varies from 100% to extremely low between host species and among populations within a host species. This variation of prevalence arises because of the host-symbiont associations due to different environmental conditions, different stages of infection history, or affected by variable genetic factors of either the symbiont or the host (Salunkhe et al. 2015). Due to manipulating its host reproductive system so that *Wolbachia* can survive in their host population despite the apparent costs of host fecundity. Cytoplasmic incompatibility (CI) inducing-*Wolbachia* causes total or partial reproductive failure between males and females with different infection statuses. It provides a reproductive advantage to female hosts, which can produce viable offspring regardless of whether they mate with infected or uninfected males (Salunkhe et al. 2015). *Wolbachia* distorts the population sex ratio by male-killing, parthenogenesis, and feminization. It promotes the production and fitness of infected females, ensuring the transmission of infection through generations. The coexistence of infected and uninfected individuals in a population occurs if there is a perfect vertical transmission of the symbiont (Duplouy et al. 2015).

Two species of the order Diptera in this study showed positive results for *Wolbachia*. *Wolbachia* infected at least 19 species of fruit flies of the genus Drosophila (Glasser and Meola, 2010). In some species, *Wolbachia* causes robust and intricate patterns of cytoplasmic incompatibility, such as *Drosophila simulans*. In contrast, in other species, reproductive phenotypes are generally weak or absent, such as *D. melanogaster*. Despite the lack of a robust reproductive phenotype, *Wolbachia* infection in *D. melanogaster* is widely spread. The mosquito species *Aedes albopictus* also showed a positive result for

Wolbachia. Another study by Ahmad et al. (2017) at eight study sites in five states (Malacca, Selangor, Terengganu, Perak, and Pahang) in Malaysia showed a high percentage of *Wolbachia* infection with 98.6% in females and 95.1% in males.

The insects in this study were collected from two areas with almost similar characters, i.e., local residential areas. The high rate of infection in the field (44,9%) indicates that *Wolbachia* thrives in open or less industrialized areas, such as residential areas, parks, and schools, which have a much higher infection rate. The infected species show similar preference patterns of food and the environment. The majority of infected species feed on decaying matter and prefer humid environments (Jeong et al. 2012). The presence of *Wolbachia* in insects in this study were widely varied among species. Age and ecology of the host, including geographical and seasonal variations, are responsible for the variation of infection rate in the natural insect population (Sarwar et al. 2017).

The *Wolbachia* surface protein (*WSP*) is commonly used as a marker for strain typing, and a strain typing system utilizing *wsp* gene has been developed. The *wsp* gene is analogous to the antigens employed for serotyping pathogenic bacteria. It is approximately ten times more variable in its DNA sequence than 16S rRNA (Zhou et al. 1998). The *wsp* gene can be detected similarly in both thorax and abdomen, and also the whole body of insect samples, indicating that *Wolbachia* was distributed equally in the whole body of insects. *Wolbachia* is distributed in the somatic and reproductive tissues of the mosquito, where transmissible pathogens reside and replicate (Zouache et al. 2009). The highest density of bacteria was found in the future oocyte, confirming a common feature of *Wolbachia*, which is to transfer from nurse cells into the oocyte through cytoplasmic dumping as in Drosophila (Ferree et al. 2005).

This study supports other findings of the ubiquity of *Wolbachia* in nearly 30-70% of the world's insect species, including flies, bees, butterflies, and mosquitoes in the different regions in the world. Considering the wide distribution of *Wolbachia* as a natural endosymbiont, artificially infecting *Wolbachia* to *Aedes aegypti* is a safe alternative method to control dengue transmission. Since public attention is focused on safety, this outcome may answer one of these crucial issues.

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