Wolbachia Enhances West Nile Virus (WNV) Infection in the Mosquito Culex tarsalis

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Abstract

Novel strategies are required to control mosquitoes and the pathogens they transmit. One attractive approach involves maternally inherited endosymbiotic Wolbachia bacteria. After artificial infection with Wolbachia, many mosquitoes become refractory to infection and transmission of diverse pathogens. We evaluated the effects of Wolbachia (wAlbB strain) on infection, dissemination and transmission of West Nile virus (WNV) in the naturally uninfected mosquito Culex tarsalis, which is an important WNV vector in North America. After inoculation into adult female mosquitoes, Wolbachia reached high titers and disseminated widely to numerous tissues including the head, thoracic flight muscles, fat body and ovarian follicles. Contrary to other systems, Wolbachia did not inhibit WNV in this mosquito. Rather, WNV infection rate was significantly higher in Wolbachia-infected mosquitoes compared to controls. Quantitative PCR of selected innate immune genes indicated that REL1 (the activator of the antiviral Toll immune pathway) was down regulated in Wolbachia-infected relative to control mosquitoes. This is the first observation of Wolbachia-induced enhancement of a human pathogen in mosquitoes, suggesting that caution should be applied before releasing Wolbachia-infected insects as part of a vector-borne disease control program.

Introduction

Efforts to control vector-borne pathogens have been hindered by evolution of insecticide resistance and failing drug therapies. Evidence suggests bed nets and indoor residual spraying with insecticides are losing efficacy in developing countries [1,2]. To improve the sustainability and efficacy of control efforts, alternative vector control strategies are being considered, including methods that suppress the pathogen instead of the vector [3,4]. Wolbachia are a genus of maternally-inherited bacterial endosymbionts that naturally occur in numerous arthropod taxa [5]. Wolbachia can inhibit viruses and parasites in fruit flies and mosquitoes [6–11] and influence reproduction of their host to facilitate spread through populations [12]. Mosquito-borne disease management programs that use Wolbachia are currently under investigation [13]. In field trials in Australia, Wolbachia reached fixation in naturally uninfected populations of Aedes aegypti [11] and the DENV blocking phenotype has been maintained [14], but the impacts of Wolbachia on reducing the incidence of disease are yet to be investigated.

Pathogen interference conferred by Wolbachia depends on various factors, including Wolbachia strain, pathogen type, infection type (natural versus artificial) and host and is not a guarantee [7,15,16]. For example, Wolbachia increases Plasmodium berghei, P. yoelii and P. gallinaceum oocyst loads in Anopheles gambiense, An. stephensi, and Aedes fluviatilis, respectively [17–19], and P. relictum sporozoite prevalence in Culex pipiens [20]. These Wolbachia-mediated pathogen enhancement studies suggest that careful examination of Wolbachia is required, since the bacterium influences insect-pathogen interactions in ways that may negatively impact pathogen control efforts.

Few studies have investigated the effect of Wolbachia on pathogen transmission by Culex mosquitoes, despite the fact they transmit viruses impacting human health [9,21,22]. Culex tarsalis is a mosquito species associated with agriculture and urban areas in the western United States [23] and is highly competent for West Nile virus (WNV), St. Louis encephalitis virus (SLEV) and western equine encephalitis virus (WEEV) [24–26]. Cx. tarsalis are naturally uninfected with Wolbachia [27]. We established Wolbachia infections in this mosquito by intrathoracic injection of purified symbionts
Author Summary

Current methods to control mosquitoes and the pathogens they transmit are ineffective, partly due to insecticide and drug resistance. One novel control method involves exploiting naturally occurring Wolbachia bacteria in insects. Wolbachia are bacterial symbionts that are attractive candidates for mosquito-borne disease control due to their ability to inhibit pathogens infecting humans. Additionally, Wolbachia affects insect reproduction to facilitate its own transmission to offspring, which has been exploited to establish the bacterium in naturally uninfected field populations. Most Wolbachia pathogen control research has focused on Aedes and Anopheles mosquitoes, but Culex mosquitoes also transmit pathogens that affect human health. We evaluated impacts of Wolbachia infection on West Nile virus (WNV) in the naturally uninfected mosquito Culex tarsalis. Wolbachia was able to efficiently establish infection in Cx. tarsalis but contrary to other studies, Wolbachia enhanced rather than inhibited WNV infection. Enhancement occurred in conjunction with suppression of Wolbachia anti-viral immune gene expression. This study indicates that Wolbachia control strategies to disrupt WNV via pathogen interference may not be feasible in Cx. tarsalis, and that caution should be used when releasing Wolbachia infected mosquitoes to control human vector-borne diseases.

Methods

Ethics statement

Mosquitoes were maintained on commercially available bovine blood using a membrane feeder. WNV infection experiments were performed under biosafety-level 3 (BSL3) and arthropod-containment level 3 (ACL3) conditions.

Mosquitoes, Wolbachia, and West Nile virus

The Cx. tarsalis YOLO strain was used for experiments. The colony was originally established from Yolo County, CA in 2009. Mosquitoes were reared and maintained at 27°C ± 1°C, 16:8 hour light:dark diurnal cycle at approximately 45% relative humidity in 30 × 30 × 30 cm cages. The wAlbB Wolbachia strain was purified from An. gambiae Sua5B cells according to published protocols [28]. Viability and density of the bacteria was assessed using the Live/Dead BacLight Kit (Invitrogen) and a hemocytometer. Viability and density of the bacteria was assessed using the Live/Dead BacLight Kit (Invitrogen) and a hemocytometer. The Viability and density of the bacteria was assessed using the Live/Dead BacLight Kit (Invitrogen) and a hemocytometer. The Viability and density of the bacteria was assessed using the Live/Dead BacLight Kit (Invitrogen) and a hemocytometer. The Viability and density of the bacteria was assessed using the Live/Dead BacLight Kit (Invitrogen) and a hemocytometer.

WNV strain WN02-1956 (GenBank: AY590222) was originally isolated in African green monkey kidney (Vero) cells from an infected American crow in New York in 2003 [29] and amplified in Aedes albopictus cells (C6/36) to a final titer of 5.0 × 10^7 PFU/mL. WNV was added to 5 mL defibrinated bovine blood (Hema-Resource & Supply, Aurora, OR) with 2.5% sucrose solution. Replicate titers were: replicate one, 8.0 × 10^7 PFU/mL, replicate two, 5.0 × 10^7 PFU/mL. Seven days post Wolbachia injection mosquitoes were fed a WNV infectious blood meal via Hemotek membrane feeding system (Discovery Workshops, Accrington, UK) for approximately one hour. Partially- or non-blood fed females were excluded from the analysis.

Fluorescence in situ hybridization (FISH) and microscopy

To characterize Wolbachia infections in Cx. tarsalis tissues, we performed fluorescence in situ hybridization (FISH) on mosquitoes at 12 dpi according to published protocols [10] with slight modifications. Briefly, mosquitoes were fixed in acetone, embedded in paraffin wax and sectioned with a microtome. Slides were dehydrated with three successive xylene washes for 5 minutes, followed by two 5-minute washes with 100% ethanol and one wash in 95% ethanol before treatment with alcoholic hydrogen peroxide (6% H2O2 in 80% ethanol) for 5 days to minimize autofluorescence. Sectioned tissues were hybridized overnight in 1 mL of hybridization buffer (50% formamide, 5 × SSC, 200 g/liter dextran sulfate, 250 mg/liter poly(A), 250 mg/liter salmon sperm DNA, 250 mg/liter rRNA, 0.1 M dithiothreitol (DTT), 0.5 × Denhardt’s solution) with Wolbachia specific probes W1 and W2 labeled with a 5-prime rhodamine fluorophore [30]. After hybridization, tissues were successively washed three times in 1 × SSC, 10 mM DTT and three times in 0.5 × SSC, 10 mM DTT. Slides were mounted with SlowFade Gold antifade reagent (Invitrogen) and counterstained with DAPI (Roche). Images were captured with a LSM 510 META confocal microscope (Zeiss) and epifluorescent BX40 microscope (Olympus). Images were processed using LSM image browsers (Zeiss) and Photoshop 7.0 (Adobe) software. No-probe, competition probe and RNase treatment controls were conducted (Figure S1).

Table 1. Primers used for qPCR.

| Primer | Sequence 5’-3’ | Reference |
|--------|----------------|-----------|
| REL1-F | GCGACTTTGGCATCAAGGCTC | This study |
| REL1-R | GTTGGACCGAGGACTGATAG | This study |
| REL2-F | GTGAGATGGCCAACAAGATG | This study |
| REL2-R | ACTCATCATATTTGGATGAGCATT | This study |
| CACTUS-F | GACCTGTGCAAGAGCTGCT | This study |
| CACTUS-R | AGTATACCACTAGCTGTC | This study |
| DEFENSIN-F | TTTGTGTTCTGCTGCTGCTT | This study |
| DEFENSIN-R | ATTCCTCACACGAAACCACAT | This study |
| DIPTERIN-F | CCCAGGGCGTCCTACTT | This study |
| DIPTERIN-R | CATGATCCAGGCGGAGAC | This study |
| ALB-GR | GGTTTTGCTTTATCAGACAAAAAG | [35] |
| ACTIN-F | GACTACCTGATGAGACTGCTGAC | [36] |
| ACTIN-R | GCAAGCTTCTGATGCTGCG | [36] |

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Vector competence for WNV

Virus infection and transmission assays were performed as described at 7 and 14 days post blood feeding [31–33]. Female mosquitoes were anesthetized with triethylamine (Sigma, St. Louis, MO), legs from each mosquito were removed and placed separately in 1 mL mosquito diluent (MD; 20% heat-inactivated fetal bovine serum [FBS] in Dulbecco’s phosphate-buffered saline, 50 ug/mL penicillin/streptomycin, 50 ug/mL gentamicin and 2.5 ug/mL fungizone). The proboscis of each mosquito was positioned in a tapered capillary tube containing 10 uL of a 1:1 solution of 50% sucrose and FBS to induce salivation. After 30 minutes, the contents were expelled into 0.3 mL MD and bodies were placed individually into 1 mL MD. Mosquito body, legs and salivary secretion samples were stored at −20°C until tested for WNV presence and Wolbachia titers. Mosquito bodies and legs were homogenized for 30 seconds utilizing Qiagen Tissue Lyser at 24 cycles/second, followed by clarification via centrifugation for one minute. Mosquito samples were tested for

Figure 1. Fluorescence in situ hybridization of Wolbachia infection in Cx. tarsalis mosquitoes 12 days post injection. Confocal microscopy of sectioned mosquitoes shows Wolbachia infection in diverse tissues after adult microinjection. A. Wolbachia localized in the abdomen of Cx. tarsalis. B. Wolbachia infection disseminated to the head and nervous tissue. C. Wolbachia is present in the muscular tissue of the mosquito. D. Wolbachia infection within and surrounding the ovarian follicles. Arrowheads denote infection within the ovarian follicle. The scale bar represents 50 um. OF; ovarian follicle, MG; midgut, FB; fat body, M; muscle, B; brain, O; omnitidia. Red = Wolbachia; Blue = mosquito DNA.
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WNV infectious particles by plaque assay on Vero cells [34]. Infection was defined as the proportion of mosquitoes with WNV positive bodies. Dissemination and transmission were defined as the proportion of infected mosquitoes with WNV positive legs and salivary secretions, respectively. Proportions were compared using Fisher’s exact test. The experiment was replicated twice.

Quantitative real-time PCR (qPCR) of *Wolbachia* density

To evaluate *Wolbachia* density in individual mosquitoes from vector competence experiments, DNA was extracted using DNeasy Blood and Tissue kits (Qiagen) and used as template for qPCR on a Rotor Gene Q (Qiagen) with the SYBR green PCR kit (Qiagen). *Wolbachia* DNA was amplified with primers Alb-GF and Alb-GR [35] and was normalized to the *Cx. tarsalis* actin gene [36] (Table 1). *Wolbachia* to host genome ratios were calculated using Qgene [37]. PCRs were performed in duplicate. Comparisons of *Wolbachia* titers between treatments were analyzed using Mann-Whitney U test.

*Cx. tarsalis* immune gene expression in response to *Wolbachia*

To explore *Wolbachia* effects on mosquito immune gene expression, one- to four- day old adult female *Cx. tarsalis* were anesthetized with CO₂ and injected as described above with *Wolbachia (wAlbB)* or Schneider’s insect media as control. Mosquitoes were provided with 10% sucrose *ad libitum* and maintained at 27°C in a growth chamber. At 7 dpi, mosquitoes were blood fed on bovine blood via glass membrane feeder. At 2 dpf, five mosquitoes per treatment were harvested and RNA extracted using RNeasy mini kits (Qiagen). Extracted RNA was DNase treated (Ambion #AM1906) and converted to cDNA using Superscript III with random hexamers (Invitrogen #18080-51) according to the manufacturers’ protocols. qPCRs were performed using the Rotor Gene Q (Qiagen) and SYBR Green qPCR kit (Qiagen) according to the manufacturer’s protocol. Five target immune genes in the Toll and IMD innate immune pathways (REL1, REL2, cactus, defensin and diptericin) were selected, primers designed based on homologous genes in the *Anopheles gambiae*, *Aedes aegypti* and *Culex pipiens* genomes and normalized to host actin (Table 1). Gene expression was analyzed by calculating ratios of target to host gene and tested for significance using Mann-Whitney U test. All qPCRs were technically replicated twice.
Results

Fluorescence in situ hybridization (FISH)

Using fluorescence in situ hybridization, we observed that \( wAlbB \) establishes an infection in both somatic and germline tissue in \( Cx. \) tarsalis 12 days post injection. \( Wolbachia \) disseminated to various tissues including the head, proboscis, thoracic flight muscles, fat body and ovarian follicles (Figure 1). \( Cx. \) tarsalis appeared heavily infected, suggesting that adult microinjection is an effective method to experimentally infect this mosquito species.

Vector competence for WNV

We evaluated the vector competence of \( Wolbachia \)-infected and uninfected \( Cx. \) tarsalis for WNV in mosquito bodies, legs and salivary secretions to determine infection, dissemination and transmission rates, respectively. Replicate results were similar, and results from pooled replicates or analysis of individual replicates were identical, so the pooled analysis is presented for clarity; results from individual replicates are available as Table S1. \( wAlbB \)-infected \( Cx. \) tarsalis displayed significantly higher WNV infection rates 7 days post-feeding (dpf) \( (P = 0.04) \). A similar but non-significant trend was observed 14 dpf (Figure 2). If mosquitoes were infected, virus dissemination and transmission rates did not differ statistically (Table S1).

Quantitative real-time PCR (qPCR) of \( Wolbachia \) density

To determine if there was a \( Wolbachia \) density effect on WNV phenotype, qPCR was used to compare \( Wolbachia \) titers in mosquitoes either positive or negative WNV. \( Wolbachia \) titers in WNV-infected versus uninfected \( Cx. \) tarsalis did not differ statistically; similarly, no significant titer differences were found in individuals that disseminated versus non-disseminated or transmitted vs. non-transmitted (Figure 3).

Discussion

Caveats of this study

It should be noted that these experiments were performed with mosquitoes transiently infected in the somatic tissues with \( Wolbachia \), rather than a stable maternally inherited infection. It remains to be seen whether a stable \( wAlbB \) infection will enhance WNV in a similar way. \( Wolbachia \) density in mosquito somatic tissues (as opposed to germline) was found to explain differences in virus infection in \( Aedes \) mosquitoes [41]. Thus, it seems likely that if stable infection in \( Cx. \) tarsalis has a similar somatic tissue distribution to a transient infection it may induce a similar virus enhancement phenotype. However, this must be tested empirically. It is also unknown whether virus enhancement is limited to WNV or occurs more broadly. Finally, we tested a single \( Wolbachia \) strain, and it is unknown whether virus enhancement is specific to \( wAlbB \) or occurs with diverse \( Wolbachia \) strains.

Previous studies have shown that pathogen suppression by \( Wolbachia \) has the potential to be a novel method for controlling vector-borne diseases [4,42–44]. Not all mosquito species are naturally infected with \( Wolbachia \), but non-infected species may
support infection once introduced and these novel infections often effectively inhibit various pathogens [3,45]. Our experiments indicate that following adult microinjection, Wolbachia is capable of establishing both somatic and germline infection in Cx. tarsalis but does not inhibit WNV infection, dissemination or transmission. In contrast, with other studies showing pathogen inhibition by Wolbachia, our data suggest that Wolbachia may in fact increase WNV infection rates in Cx. tarsalis, particularly at early time points. Increased early infection has the potential to shorten the extrinsic incubation period of the pathogen, which can dramatically increase the reproductive rate of the virus [19]. It has become increasingly clear that Wolbachia does not always suppress pathogens in insects [46]. For example, the cereal crop pest Spodoptera exempta is more susceptible to nucleopolyhedrovirus mortality in the presence of Wolbachia [47]. In the mosquitoes An. gambiae An. stephensi, Ae. flavidus and Cx. pipiens, Wolbachia enhances Plasmodium berghei, P. yoelii, P. gallinaceum and P. relictum, respectively [17–20]. Enhancement may be dependent on the host-Wolbachia strain-pathogen system of interest, as Wolbachia strains that block one pathogen yet enhance another have been documented [9,17]. Wolbachia-mediated pathogen enhancement may be a common yet often ignored phenomenon, which merits attention when designing Wolbachia-based strategies for disease control [46].

Intracellular infection with bacteria may alter the cellular environment in multiple ways, including bacterial manipulation to avoid host immune defenses [48]. Though the exact Wolbachia-mediated inhibition mechanism is unknown, studies have suggested that Wolbachia indirectly modulates mosquito immunity [40,49]. Wolbachia can activate the Toll pathway, stimulating a cascade of events that have been correlated with inhibition of dengue and Plasmodium in mosquitoes [39,50,51]. In contrast, in Cx. tarsalis, wAlB infection significantly downregulated REL1 (the activator of the Toll pathway), suggesting that in this system Wolbachia may down regulate antiviral Toll-based immunity leading to increased virus infection. However, while statistically significant, this decrease in REL1 expression was modest, and further study is required to determine the precise mechanism of Wolbachia-based WNV enhancement in this system.

To our knowledge this is first study showing Wolbachia can potentially enhance a vector-borne pathogen that causes human disease. Our results, combined with other Wolbachia enhancement studies [17–20,46–47], suggest that field deployment of Wolbachia-infected mosquitoes should proceed with caution. Wolbachia effects on all potential pathogens in the study area should be determined. Additionally, several studies have shown that Wolbachia is capable of horizontal transfer to other insect species which could have unforeseen effects on non-target insects [52–54]. A lack of understanding of Wolbachia-pathogen-mosquito interactions could impact efficacy of disease control programs. Cx. tarsalis is a competent vector for many human pathogens, and further studies that assess alternative Wolbachia strains and viruses in Cx. tarsalis may elucidate the importance of host background on pathogen interference phenotypes in this medically important mosquito species.

**Supporting Information**

**Figure S1 FISH controls.** Red: Wolbachia, Blue: mosquito DNA. Green: background fluorescence. Top row: positive (wAlB) control. (PDF)

**Table S1 Results from individual vector competence replicates.** (PDF)

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**Author Contributions**

Conceived and designed the experiments: BLD GLH LDK JLR. Performed the experiments: BLD GLH OP ACM. Analyzed the data: BLD GLH JLR. Contributed reagents/materials/analysis tools: GLH LDK JLR. Contributed to the writing of the manuscript: BLD GLH LDK JLR.

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