J Vector Borne Dis 56, June 2019, pp. 92–97

Research Articles

Pilot survey of mosquitoes (Diptera: Culicidae) from southeastern Georgia, USA for *Wolbachia* and *Rickettsia felis* (Rickettsiales: Rickettsiaceae)

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ABSTRACT

Background & objectives: Mosquito surveillance is one of the critical functions of local health departments, particularly in the context of outbreaks of severe mosquito-borne viral infections. Unfortunately, some viral and parasitic infections transmitted by mosquitoes, manifests non-specific clinical symptoms which may actually be of rickettsial etiology, including *Rickettsia felis* infections. This study tested the hypothesis that mosquitoes from southeastern Georgia, USA may be infected with *Rickettsia felis* and *Wolbachia,* an endosymbiotic bacterium of the order Rickettsiales.

Methods: Specimens of the five most common mosquito species occurring in the region were collected using gravid and light-traps and identified using morphological keys. Mosquitoes were then pooled by species, sex, trap and collection site and their DNA was extracted. Molecular methods were used to confirm mosquito identification, and presence of *Wolbachia* and *R. felis*.

Results: Wolbachia DNA was detected in 90.8% of the mosquito pools tested, which included 98% pools of *Cx. quinquefasciatus* Say (Diptera: Culicidae), 95% pools of *Ae. albopictus* Skuse (Diptera: Culicidae), and 66.7% of pools of *Cx. pipiens* complex. Samples of *An. punctipennis* Say (Diptera: Culicidae) and *An. crucians* Wiedemann (Diptera: Culicidae) were tested negative for *Wolbachia* DNA. Three genotypes of *Wolbachia* sp. belonging to Group A (1 type) and Group B (2 types) were identified. DNA of *R. felis* was not found in any pool of mosquitoes tested.

Interpretation & conclusions: This study provides a pilot data on the high presence of *Wolbachia* in *Cx. quinquefasciatus* and *Ae. albopictus* mosquitoes prevalent in the study region. Whether the high prevalence of *Wolbachia* and its genetic diversity in mosquitoes affects the mosquitoes' susceptibility to *R. felis* infection in Georgia will need further evaluation.

Key words BioB gene; Georgia; mosquito; *Rickettsia; Wolbachia;* 16S rRNA gene

INTRODUCTION

Mosquitoes are the most dominant group of blood-sucking ectoparasites responsible for transmission of numerous viral, bacterial, and parasitic diseases of humans and other vertebrates[1]. The State of Georgia, USA is endemic for many species of mosquitoes including *Culex quinquefasciatus* Say (Diptera: Culicidae), *Aedes albopictus* Skuse and *Ae. aegypti* Linnaeus[2]. Mosquito surveillance is one of the critical functions of state and local health departments, particularly in the context of recent or potential outbreaks of severe mosquito-borne viral infections, including West Nile virus, dengue, chikungunya, and Zika. It should be emphasized that some viral and parasitic infections manifest with several non-specific clinical symptoms which may in fact be of rickettsial etiology, including *R. felis* infections[3–5]. Such situations are known in Mexico, Africa, and Sri Lanka; these areas are affected by many concurrent tropical diseases presenting with febrile syndrome[3–5]. Georgia, USA, is a state which was previously an epicenter of flea-borne rickettsiosis and where *R. felis* and cat fleas are still very abundant[6]; however, mosquitoes in the United States have not been screened for presence of *R. felis*. This situation is in contrast to recent reports that mosquitoes, including *Anopheles*, *Aedes* and *Culex* spp., from Gabon, Côte d'Ivoire and China are PCR positive for *R. felis* DNA[7–9]. Furthermore, *Anopheles gambiae* Giles (Diptera: Culicidae) has been shown to transmit *R. felis* in an experimental setting[10]; these findings implicate mosquitoes as potential unrecognized vectors for this pathogen.

Many natural populations of mosquitoes are also infected to various degrees with *Wolbachia pipientis*, a maternally inherited gram-negative endosymbiotic bac-

terium of the order Rickettsiales[11]. *Wolbachia* are known for reproductive parasitism; manipulation of host reproduction gives *Wolbachia* a means of genetic drive and permits rapid spread through uninfected insect populations. *Wolbachia* infections in mosquitoes are not ubiquitous and are dependent on the strain, the host and other pathogens present. Naturally infected *Ae. albopictus*, a vector of dengue virus and *Culex pipiens* Linnaeus (vector of West Nile virus) typically have a low *Wolbachia* density, and seldom exhibit any sign of severe manipulation of populations by *Wolbachia*[12]. *Wolbachia* in *Ae. aegypti* inhibits dengue infection, and decreases vector competence as a result of the pathogen interference phenomenon[12]. Understanding how different mosquito-borne *Wolbachia* strain-host combinations interact with various pathogens and whether any derivative effects on the pathogens occur, has become a vital part in *Wolbachia*-based control of mosquito-transmitted diseases.

Recent microbiome studies reported a very low abundance (0.35%) of *Wolbachia* in larvae of *Ae. albopictus* and *Cx. quinquefasciatus* collected in Athens, Georgia[13]. *Wolbachia* has been also detected in other blood sucking ectoparasites collected across the State of Georgia (USA), including *Amblyomma americanum* Linnaeus (Akari: Ixodida)[14], and *Ctenocephalides felis* Bouché (Siphonaptera) and *C. canis* Curtis which are the main vectors of *R. felis*[15]. The purpose of this study was to examine infection rates with *Wolbachia* and *R. felis* in mosquitoes prevalent in southeastern Georgia, USA. The City of Statesboro is one of the new sentinel surveillance sites funded by the State of Georgia due to concerns about expansion of Zika virus.

MATERIAL & METHODS

Collection and identification

Mosquitoes were collected from four locations in Statesboro, Georgia (32.4488 °N, 81.7832 °W) using four gravid traps [model 1712, John W. Hock Company, Gainesville, Florida (FL), USA] and four CDC miniature light-traps (model 512, John W. Hock Company, Gainesville, FL) on August 11, 2016 as a part of the city trapping/surveillance project. The trapping sites were primarily in residential areas of the city with flat to gentle sloping topography and with vegetation ranging from minimal canopy and typical household shrubbery, to sites adjacent to large suburban forests, a church and elementary school. One site was at the city's public works facility adjacent to a large drainage ditch and suburban forest. All traps were set in the evening between 1730 and 1900 hrs and retrieved the next morning between 1000 and 1200 hrs. On the day the traps were set, the weather was mostly cloudy with a low/high temperature of 22.8 to 31.7 °C; while on the next day it was fair with a temperature ranging from 23.3 to 33.3 °C. During three days prior to setting the traps, it rained at various intervals.

Mosquitoes were identified using morphological keys[16–17]; and 2–11 mosquitoes were pooled based on species, sex, trap, and collection site. Heads were removed from each specimen to prevent inhibition of PCR amplification[18]. DNA of mosquitoes was extracted using DNeasy Blood and Tissue Kit [Qiagen, Valencia, California (CA)] and stored at 4 °C prior to testing. Identification of *Cx. pipiens* complex mosquitoes was confirmed by detecting a fragment length polymorphism in the second intron of the *Ace-2* nuclear gene according to the protocol of Smith and Fonseca[19].

PCR detection of Rickettsia felis and Wolbachia

Detection of *R. felis* DNA was performed using a TaqMan assay targeting the species-specific BioB gene[20]. Each reaction was set up using 4 µl of DNA, 10 pmol (final amount per reaction) of each forward (RF_BioBF: 5'-ATGTTCGGGCTTCCGGTATG-3') and reverse (RF_BioR: 5'-CCGATTCAGCAGGTTCTTCAA-3' primers, 5 pmol probe (5'-6-FAM-GCTGCGGCGGTATTTTAG-GAATGGG-TAMRA-3'), and Brilliant III Master Mix (Agilent, Santa Clara, CA). Sterile DNase and RNase free-water was used as a negative control for each reaction; recombinant plasmid containing an insert of the *R. felis* BioB gene fragment was used as a positive control. PCR amplification and subsequent analysis was performed using a BioRad CFX96 Instrument (BioRad, Hercules, CA).

Detection of *Wolbachia* DNA was performed using a TaqMan assay targeting the 16S rRNA gene of *Wolbachia,* which was developed for the purpose of this study. The forward (WN16S-F: 5'-CACAGAAGAAGTCCTGGC-TAAC-3') and reverse (WN16S-R: 5'-CGCCCTTTAC-GCCCAATAA-3') primers and probe (WN16S-probe: 5'-HEX-CGGTAATACGGAGAGGGCTAGCGTTA-BHQ1-3') combinations were selected based on a consensus region identified from a multiple sequence alignment of 16S rRNA gene sequences of *Wolbachia* downloaded from NCBI GenBank and synthesized by Eurofin Genomics (Louisville, Kentucky). Each reaction was set up using 1.5 µl of DNA, 3.75 pmol of each forward and reverse primer and 1.5 pmol probe, and Brilliant III Master Mix (Agilent). After initial denaturation at 95 °C for 2 min, amplification consisted of 45 cycles of denaturation at 95 °C for 3 sec followed by annealing and elongation at 55 °C for 30 sec. Sterile DNase and RNase free-water was used

as the negative control for each reaction, and DNA from two-lined spittlebug *Prosapia bicinecta* Say (Hemiptera: Cercopidae) containing *Wolbachia* DNA was used as the positive control.

Genotyping of Wolbachia

Wolbachia genotyping was performed using PCR amplification of *wsp* according to a previously described protocol[21] with slight modification. Primary amplification was performed using primers wsp81F (5'-TGGTCCAATAAGTGATGAAGAAAC-3') and wsp691R (5'-AAAAATTAAACGCTACTCCA-3') followed by two semi-nested PCR amplifications using genotype specific primers. Primers wsp328F (5'-CCAGCAGATACTATTGCG-3') and wsp691R were used to amplify 363-bp fragment of *Wolbachia* genotype A. Primers 183F (5'-AAGGAACCGAAGTTCATG-3') and 691R were used to amplify a 508-bp fragment of *Wolbachia* genotype B. All the PCR were set up using 2 µl of DNA, 40 pmol of each forward and reverse primers and Taq PCR Master Mix (Qiagen). PCR cycling conditions consisted of original denaturation for 5 min at 95 °C, followed by 35 cycles of 30 sec at 95 °C, 30 sec at 55 °C, 30 sec at 68 °C, and completed by final extension for 5 min at 72 °C. PCR products were separated on a 1% agarose gel for 30 min at 80 volts, stained with ethidium bromide and observed under UV-light.

Sequencing

Amplicons were purified using the Monarch® PCR and DNA Cleanup kit following the manufacturer's recommendations (New England Biolabs, Ipswich, Massachusetts). DNA concentration in purified samples was determined using a Qubit 3.0 Fluorometer (Life Technologies Invitrogen, Carlsbad, CA). Samples were sequenced using Sanger sequencing (Clemson University Genomics Institute, Clemson, South Carolina). Sequences were analyzed for quality, edited, and contigs were assembled using Sequencher 5.4.1 (Gene Codes Corporations, Ann Arbor Michigan). Sequence identity was determined using the Basic Local Alignment Search Tool (BLAST) in GenBank. New sequences generated in this study were submitted to NCBI GenBank with accession numbers MG765532–MG765534.

Statistical analysis

Confidence intervals (CI) for minimal infection rate (MIR) and maximum likelihood estimate (MLE) for infection rate (IR) were calculated using CDC's mosquito surveillance software for pooled data[22].

RESULTS

Overall, 552 mosquitoes were collected and identified using morphological criteria as *Ae. albopictus* (101), *An. punctipennis* Say (10), *An. crucians* Wiedemann (2), *Cx. quinquefasciatus* (407) and *Cx. pipiens* (32). PCR amplification of the *ace*-2 locus fragment from 47 pools of mosquitoes identified as *Cx. quinquefasciatus* produced a single fragment of 274-bp thus confirming their identification. There were 2 amplicons of 247-bp and 610-bp amplified from DNA of three mosquito pools which were morphologically identified as *Cx. pipiens* based on their lighter color and environmental conditions at the trapping site. Accordingly, a double-band profile was attributed to the presence of *Cx. quinquefasciatus* and *Cx. pipiens* hybrids; however, an accidental pooling of the two species can't be excluded. For the purpose of this report those will be identified as *Cx. pipiens* complex since molecular identification was not done on individual specimens. *Aedes albopictus* and *Cx. quinquefasciatus* were present at all the four sites, *An. punctipennis* were collected at two locations, and *Cx. pipiens* complex mosquitoes were found only at one location.

A summary of the testing results is presented in Table 1. DNA of *Wolbachia* was detected in 90.8% (n =

Table 1. Summary of TaqMan detection of *R. felis* and *Wolbachia* in mosquitoes collected

Collection			TaqMan assay result				MIR	MLE for IR
Mosquito species	No. of pools		*R. felis*	*Wolbachia* +(ve) pools				
	Male	Female	Total (+)ve	Male	Female	Total (+)ve (Prevalence)		
Cx. quinquefasciatus	5	42	0/47	5/5	41/42	46/47 (97.9)	8.02, 14.04	34.48, 100
Ae. albopictus	11	11	0/22	11/11	10/11	21/22 (95.5)	12.9, 28.7	40.5, 91.9
Cx. pipiens Complex	0	3	0/3	NA	2/3	2/3 (66.7)	0, 21.1	2.26, 41.35
An. punctipennis	1	2	0/3	0/1	0/2	0/3 (0)	NA	NA
An. crucians	0	1	0/1	NA	0/1	0/1 (0)	NA	NA
Total	17	59	0/76	16/17	53/59	69/76	9.96, 15.59	36.94, 67.93

Numerators corresponds to the number of mosquito pools tested positive in TaqMan assay and denominator corresponds to the total number of mosquitoes tested; NA—Not applicable; MIR—Minimal infection rate (per 100) for pooled sample data; MLE for IR—Maximum likelihood estimate for infection rate. Both are at 95% confidence level; Figures in parentheses indicate percentages.

76) of the mosquito pools tested, which included 46/47 (97.9%) pools of *Cx. quinquefasciatus,* 21/22 (95.5%) pools of *Ae. albopictus*, and 2/3 (66.7%) pools of *Cx. pipiens* complex. Samples of *An. punctipennis* and *An. crucians* tested negative for *Wolbachia* DNA. Estimated MIR per 100 mosquitoes ranged from 9.96 to 15.59% (95% CI). Bias corrected MLE for infection rate ranged from 36.94 to 67.93% (95% CI). There were no statistically significant differences in MIR between collections sites (p >0.05). DNA of *R. felis* was not detected in any of the 76 mosquito pools (comprising 552 mosquitoes) tested.

Sequencing of the PCR amplicons generated using specific primer sets was used to determine the genetic types of *Wolbachia* infecting 11 *Cx. quinquefasciatus* and 12 *Ae. albopictus* mosquito pools. All the *Cx. quinquefasciatus* tested were positive for B genotype of *Wolbachia*. Nucleotide sequences of the amplicons generated from *Cx. quinquefasciatus* (MG765534) were most similar to the wPip *wsp* type (AF020060, AF020061) previously detected in *Cx. pipiens* from Tunisia and *Cx. quinquefasciatus* from Gainesville, Florida. For *Ae. albopictus,* 9/12 pools were positive for A genotype and 10/12 pools for B genotype. Nucleotide sequences of the analyzed amplicons were most similar to the homologous *wsp* sequences of *Wolbachia* sp. WalbA and WalbB (GenBank: MG765532 and MG765533, respectively) both, previously identified in *Ae. albopictus* (GenBank: AF020058, AF020059) from Houston, Texas.

DISCUSSION

A total of five species of mosquitoes, endemic to Georgia, were examined in this study, *viz. Cx. quinquefasciatus, Cx. pipiens* complex*, Ae. albopictus, An. punctipennis* and *An. crucians*. *Culex quinquefasciatus* was the most prevalent species; mosquitoes assigned to *Cx. pipiens* complex had a similar light appearance and were from an area surrounded with some standing water of clear appearance and less murky or eutrophic than other sites[23]. These specimens potentially could be the hybrids of *Cx. quinquefasciatus* and *Cx. pipiens* as demonstrated by *ace*-1 restriction profile. The City of Statesboro, USA is located (32.4488 °N, 81.7832 °W) within the geographic limits of 30 and 40 °N, delineating the hybrid zone between these two species based on recent microsatellite studies[24–25]. Hybrids of *Cx. pipiens* and *Cx. quinquefasciatus* mosquitoes are known to exhibit different host preference which may affect the patterns of pathogen transmission[19, 24–25].

All the mosquitoes tested PCR negative for *R. felis;* however, most mosquito pools tested PCR positive for *Wolbachia*. The lack of detection of *R. felis* DNA in mosquitoes from Georgia is in contrast to findings previously reported from Côte d'Ivoire, Gabon and China [7–9]. In those studies, 1.3% (n = 77) *An. gambiae* from Côte d'Ivoire[8], 3.1% (n = 96) *Ae. aegypti* from Gabon[7], as well as 5.4% (n = 428) of *An. sinensi* (6.25%, n = 32) and *Cx. pipiens pallens* (5.5%, n = 396) from Jiangsu, China tested PCR positive for *R. felis9*. Furthermore, a closely related *Rickettsia* sp. has been detected in *An. gambiae* from these places[7–9], and recently *Rickettsia* sp. A12.2646, A12.2638 and A12.3271 have been identified using multiple locus sequencing from *Mansonia uniformis*, *Cx. pipiens*, and *Ae. esoensis* from Korea[26].

Since, all PCR controls for *R. felis* DNA performed as expected, it can be said that the negative PCR results indicate the absence of *R. felis* DNA in association with the mosquito pools tested in this study. This suggests that these mosquitoes, mostly trapped in urban locations, did not feed on animals which maintain *R. felis* rickettsemia. Virginia opossums (*Didelphis virginiana*) are considered to be the main reservoirs of *R. felis* in areas endemic for this pathogen in the USA. Opossums are common in Georgia and can be frequently seen in residential areas, and *R. felis* is highly prevalent in *C. felis* collected from Georgia[6]. *Culex quinquefasciatus* and *Ae. albopictus* were the most prevalent species examined in this study; *Cx. quinquefasciatus* is known for its preferential feeding on humans and dogs, but with only infrequent feeding on cats and opossums[27]. *Aedes albopictus* has been reported to exhibit an opportunistic host-feeding pattern with a predilection to feed on mammalian hosts, with most blood meals from humans, followed by dogs and cats, but only rarely on opossums[28–29]. In contrast, studies performed in the neighbouring State of North Carolina, USA demonstrated frequent feeding of *Cx. pipiens* on opossum bloods in 55% of the samples tested[29]; however, *Cx. pipiens* was not prevalent in present collection. Similarly, *Anopheles* mosquitoes, reported *R. felis*-positive in other studies were not common among mosquitoes collected for this study.

The TaqMan assay used in this study for detection of *R. felis* is species-specific but it may amplify DNA of closely related *R. asemboensis* as determined by BLAST against its genome sequence. Detectability of "*Candidatus* Rickettsia senegalensis" and other *R. felis*-like agents potentially present in the same areas can't be predicted due to the uncultivated status of those rickettsiae, and the limited number of sequence targets available for these organisms in GenBank, mostly more conserved targets such as *omp*B, *glt*A or 17-kDa protein antigen gene. Although, it would be advantageous to have a broader detection or multiplex assay permitting simultaneous detection of *R.*

felis and its nearest relatives, their prevalence is reported to be much lower when compared to that of *R. felis*[30–31].

DNA of *Wolbachia* was detected in 66.7 to 97.9% pools of *Culex* mosquitoes and 95.5% of *Ae. albopictus*; however, DNA of *Anopheles* species tested negative for *Wolbachia*. The occurrence of *Wolbachia* A and B supergroups was identified based on sequencing of the *wsp* gene, the original typing system developed for *Wolbachia*[21]. Most recently multilocus sequence typing (MLST) based on sequencing of five genes has been developed and its usefulness has been demonstrated for discriminating between closely related strains, consequently, this typing provides more information for comparative genetics and molecular evolution of diverse genogroups of *Wolbachia*[32]. The relationships inferred using MLST and *wsp* sequences are complementary; furthermore, *C. pipiens* strains studied by Baldo *et al*[32] had identical MLST and *wsp* profiles suggesting that the use of *wsp* genotyping is adequate for the purpose of this pilot study. The MLST should be implemented as a part of future surveillance projects to confirm the preliminary findings using *wsp* and to obtain more information about the extent of the genetic diversity of *Wolbachia* in individual mosquitoes circulating in the region.

Wolbachia pipientis is a maternally inherited endosymbiotic bacterium which is found in a large variety of insects and other arthropods, and filarial nematode populations[11]. Being found in different somatic and reproductive tissues of arthropods, *Wolbachia* can cause a variety of reproductive alterations, may affect the arthropod's longevity, and can also exhibit an exclusion interference with pathogens in the arthropod[12]. These properties suggested the concept of utilizing *Wolbachia* as a biological vector-control strategy to reduce pathogen transmission by mosquitoes, particularly emerging arboviruses[33–34]. Geographically divergent populations, biotypes and species of mosquitoes exhibit significant variation in infection with *Wolbachia,* ranging from complete absence to 80–100% prevalence[35–37]; this suggests that different mosquito populations may have different susceptibilities and/or protection against viruses or other pathogens associated with differences in *Wolbachia* carriage. Earlier studies conducted with laboratory maintained *An. gambiae* demonstrated horizontal transmission of *R. felis*. *Rickettsia felis* was visualized by immunofluorescence in salivary glands, in and around the gut, and in the ovaries of infected mosquitoes, and detected in faeces; however, no vertical transmission was observed[10]. This suggests an occurrence of possible exclusion interference effect (due to *Wolbachia* infection) in mosquitoes; however, this speculation needs further experimental confirmation and a better understanding of the genetic structure and diversity of *Wolbachia* genotypes present in mosquitoes, endemic to Georgia to determine if those mosquito strains of *Wolbachia* can inhibit acquisition of pathogens in the state.

CONCLUSION

The result of the study indicates high prevalence of *Wolbachia* in *Cx. quinquefasciatu*s and *Ae. albopictus* mosquitoes collected from Georgia, USA. Large sampling of under-represented mosquito species is needed to determine the regional presence of *Wolbachia* in these vectors. Further, studies are warranted to define the genetic diversity of circulating *Wolbachia* and to assess its variability within and among wild mosquito populations in Georgia.

Conflict of interest: The authors declare no conflict of interest.

Ethical statement: Not applicable.

ACKNOWLEDGEMENTS

Funding for this project was provided by 2013 and 2015 Georgia Southern University Faculty Research Awards to Marina Eremeeva. Matthew Anderson was supported by the 2016 American Society for Microbiology Undergraduate Research Fellowship, and Georgia Southern University's College of Science and Mathematics COUR grant. The authors thank Dr Gregory A. Dasch (Centers for Disease Control and Prevention, Atlanta) for reviewing and providing helpful suggestions to improve this manuscript.

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Received: 18 June 2018 *Accepted in revised form:* 15 November 2018