Molecular Evidence of high prevalence of Wolbachia Species in Wild-Caught Aedes albopictus and Aedes aegypti mosquitoes on Bioko Island, Equatorial Guinea

Johanna N. Giger
Swiss Tropical and Public Health Institute
Salome Hosch
Swiss Tropical and Public Health Institute
Nele G. von Planta
Swiss Tropical and Public Health Institute
Nestor Rivas Bela
Medical Care Development Global Health
Godwin Fuseini
Medical Care Development Global Health
Raul Nguema Ncogo Oyana
Medical Care Development Global Health
Mathias Leonard Houag
Medical Care Development Global Health
Prudencio Bibang Engono Efiri
Medical Care Development Global Health
Valeriano Oluy Nsue Maye
Medical Care Development Global Health
Guillermo A. García
Medical Care Development Global Health
Wonder P. Phiri
Medical Care Development Global Health
Tobias Schindler
University of Basel
Claudia Daubenberger
claudia.daubenberger@swisstph.ch

University of Basel

Short Report

Keywords: Bioko Island, Aedes albopictus, Aedes aegypti, Wolbachia spp., qPCR, metagenomics

Posted Date: March 13th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-4046525/v1

Additional Declarations: No competing interests reported.

Abstract

Background: Distribution of *Aedes albopictus* and *Aedes aegypti* are currently unknown on Bioko Island Equatorial Guinea.

Methods: *Aedes* spp. were collected between February 2020 and August 2021 on Bioko Island as a by-product of malaria vector entomological surveillance. The mosquitoes were transferred to Switzerland. Utilizing qPCR (quantitative polymerase chain reaction) on extracted nucleic acids, the mosquitoes were distinguished between *A. albopictus* and *A. aegypti*. Subsequently, the host species of their last blood meal was identified. Metagenomic analyses of potential pathogens were performed using Oxford Nanopore sequencing. A qPCR assay to detect Wolbachia was developed and used to screen *Aedes* spp.

Results: *A. albopictus* represents the dominant *Aedes* species on Bioko Island. *Aedes* spp. mosquitoes predominately fed on humans, few *A. albopictus* positive pools additionally contained blood meals from dog or chicken. Eight out of nine tested *Aedes* spp. pools contained DNA sequences that matched to *Wolbachia* spp. assessed by metagenomics. The remaining 72 pools were tested by qPCR for presence of *Wolbachia* spp. DNA. In total, 87% of all samples tested were positive for *Wolbachia* spp. *A. albopictus* seemed to be significantly more affected by *Wolbachia* spp. than *A. aegypti*.

Conclusions: *A. albopictus* has become the dominant *Aedes* species on Bioko Island and can be found in all four districts. *Wolbachia* spp. endosymbionts seem to be more prevalent in *A. albopictus* compared to *A. aegypti*. The impact of *Wolbachia* spp. presence on potential transmission of Flaviviruses in these different local mosquito populations warrants further investigation.

Background

Mosquito species belonging to the genus *Aedes* are one of the most important disease vectors besides *Anopheles* globally [1, 2]. Emerging and re-emerging Flaviviruses like Yellow Fever virus (YFV), Dengue virus (DENV), Chikungunya virus (CHIKV) and Zika virus (ZIKV) are transmitted by *Aedes* spp. [3, 4]. *Aedes aegypti* and *Aedes albopictus* are mostly found in tropical to subtropical regions worldwide. In March 2022, the World Health Organisation (WHO) launched the Global Arbovirus Initiative as a strategic plan to tackle arboviruses with epidemic potential [5]. The epidemiology of arboviruses and the resulting arboviral disease prevalence and clinical load is currently only partially known in Central and West Africa [6, 7]. Suspected *Aedes*-borne virus outbreaks seem to have increased in recent years in West Africa [6]. A number of larger outbreaks of DENV and CHIKV in West Africa have been described in urban areas of Burkina Faso [8], and Cote d'Ivoire [9] and CHIKV outbreaks in Gabon [10] and Sierra Leone [11]. Importantly, fever attributable to arbovirus infections might lead to an overestimation of clinical malaria in Sub-Saharan African countries resulting in antimalarial drug overuse [12].

Enhanced regular surveillance of *Aedes* spp. populations to monitor the effect of climate change and rapid urbanisation is essential to understand pathogens transmitted and disease outbreaks caused [13, 14]. This entails monitoring of georeferenced *Aedes* spp. distribution, observation of population

dynamics over time and establishment of infection rates with different pathogens transmitted [15]. The continuous collection of this information is crucial for early detection, timely response, thereby reducing the public health impact of arbovirus disease outbreaks.

Targeted vector control strategies may include the use of insecticides, biological control methods, or community engagement to reduce mosquito breeding sites. One possible biological control method makes use of the naturally occurring endosymbiont *Wolbachia* spp., which inhabits the intestines of *Aedes* mosquitoes. Arthropods infected with *Wolbachia* spp., show a change in mating behaviour (e.g. cytoplasmic incompatibility) and a lower transmission rate of virus infections [16, 17, 18].

We have collected 557 *Aedes* spp. mosquitoes on Bioko Island in the years 2020 and 2021 and analysed with molecular tools the species distribution of *A. albopictus* and *A. aegypti*. The results of our study indicate that the ratio of *A. albopictus* and *A. aegypti* shifted over the last decade and that *A. albopictus* became the predominant species on Bioko Island. *A. albopictus* displayed an increase carriage of *Wolbachia* spp. compared to *A. aegypti* with 87% of all mosquito samples testing positive for this endosymbiont. This report is the first time that the distribution of *Wolbachia* spp. in the Aedes population is described in Equatorial Guinea.

Methods

Study area and mosquito collection

Aedes spp. were collected at sentinel sites defined in urban, peri-urban, and rural landscapes across Bioko Island. Collections were performed using Human Landing Catch (HLC) and Centre for Disease Control Light Trap (CDC-LT) methods as a by-product of the routine entomological surveillance for malaria vectors between February 2020 and August 2021 [19]. Mosquitoes were collected throughout the night from seven PM to six AM the following morning in two houses randomly selected per sentinel monitoring site and per method. One volunteer each was placed indoors and outdoors of selected houses in a four person/night catch. Simultaneously, two CDC light traps each were installed indoors and outdoors of two other randomly picked houses. Resulting again in a four trap/night catch. *Aedes* spp. collected via HLC were preserved according to hour and place of collection in Eppendorf tubes, while *Aedes* spp. collected via CDC-LT were pooled in groups of ten per collection place. All mosquitoes were stored in DNA/RNA Shield (Zymo Research, USA) at room temperature until shipped to Swiss TPH in Allschwil, Switzerland, for further analysis.

Sample pooling and nucleic acid extraction

Out of 233 tubes received that contained between one to 14 individual mosquitoes, 30 tubes were extracted as individual samples derived from the same night, same site and same collection method. The remaining 194 tubes were grouped into 51 pools by collection district and whenever possible by sentinel collection site and collection year. The 30 vials extracted individually contained between one and seven

Aedes spp. mosquitoes (median of two mosquitoes). The 51 pools contained between two and 29 mosquitoes (median of nine mosquitoes). For nucleic acid extraction, mosquitoes were homogenized for 12 minutes on a Disruptor Genie (Scientific Industries, USA) using stainless steel beads with a diameter of five mm (Qiagen, USA), followed by nucleic acid extraction using the QIAamp Viral RNA Mini Kit (Qiagen, USA) according to manufacturer's protocol.

General qPCR settings

All qPCR reactions were set up as follows if not indicated otherwise: The qPCR and (RT-qPCR) assays were run on a Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, California, USA). Each qPCR reaction consisted of 1x Luna Universal Probe One-Step Reaction Mix (New England Biolabs), primer and probe mix and two µL of extracted nucleic acids in a total volume of 10 µL. The reverse transcription qPCR (RT-qPCR) runs were set up with the same master mix as the qPCR runs and complemented with 1x Luna WarmStart RT Enzyme Mix (New England Biolabs, USA). Samples were analysed in duplicate. In each qPCR run, non-template (molecular biology grade H_2O) controls and controls positive for the target were included. The controls had to be non-template control, respectively positive to be encountered as valid. The runs were set up according to Table 1 whereas denaturation and annealing/ extension steps were repeated 45 times.

Table 1: Conditions that were used for RT-qPCR runs. It was screened for the pathogens belonging to the family Flaviviridae, dengue serotypes 1 – 4 (DENV), Chikungunya virus (CHIKV), Rickettsia spp., Yellow Fever virus (YFV) and Zika virus (ZIKV).

Experiment	RT-Step		Heat Activation		Denaturation		Annealing/Extension	
Extraction control RT-qPCR	10 min	55 °C	1 min	95 °C	15 s	95 °C	45 s	60 °C
***Aedes* species identification**	-	-	3 min	95 °C	15 s	95 °C	45 s	60 °C
Identification of *Aedes* spp. transmitted human pathogens								
Pan-Flaviviridae	5 min	50 °C	1 min	95 °C	3 s	95 °C	30 s	60 °C
DENV-1 - DENV-4	5 min	50 °C	1 min	95 °C	3 s	95 °C	30 s	60 °C
CHIKV, *Rickettsia* spp., YFV and ZIKV	5 min	50 °C	1 min	95 °C	3 s	95 °C	30 s	60 °C
Blood feeding of *Aedes* spp.	10 min	55 °C	3 min	95 °C	15 s	95 °C	45 s	59 °C
Screening for *Wolbachia* spp.	-	-	3 min	95 °C	15 s	95 °C	45 s	56 °C

Extraction control RT-qPCR

For quality control and quantification of the extracted RNA and DNA, a quantitative PCR assay (qPCR) targeting the 18S and 28S ribosomal RNA/DNA genes of *Aedes* spp. was used (see Additional file 1: Table S1). In addition, human RNaseP (HsRNaseP) was used to detect mosquito feeding on humans.

Aedes species identification

To distinguish *A. albopictus* and *A. aegypti*, a qPCR assay that targets a fragment of the *A. aegypti* gene *AAEL017129*, and a fragment of the *A. albopictus* gene *AALF027084* was performed.

Identification of *Aedes* spp. transmitted human pathogens

To detect human pathogens, three different multiplex RT-qPCR assays were used. The first assay targets the *NS5* gene of viruses belonging to the family Flaviviridae. The second assay was performed to detect and distinguish all four dengue virus serotypes (DENV-1, DENV-2, DENV-3, DENV-4). The last multiplex assay was used to screen for genetic material of CHIKV, *Rickettsia* spp., YFV and ZIKV. For all three assays, 1x Fast Virus 1-Step Master Mix (Applied Biosystems™, Thermo Fisher Scientific Inc) instead of Luna Universal Probe One-Step Reaction Mix was used.

Blood feeding of *Aedes* spp. identification

A RT-qPCR was used to test all mosquito samples for the source of the last blood meal. The mosquitoes were screened for presence of dog, cat and rat DNA targeting the cytochrome B gene. To screen for the presence of bird DNA, markers for 16S genes and an intergenic region specific for mice were used (Additional file 1: Table S1).

Sanger sequencing

The PCR product of samples positive for the avian primers were sent to Microsynth AG (Switzerland) for confirmatory Sanger sequencing. PCR was performed with the pan-avian qPCR primers to generate a 96 bp long amplicon of the 16S region.

Oxford Nanopore sequencing

Metagenomic sequencing of all nucleic acids was done for nine *Aedes* spp. pools according to a published protocol [20]. Reverse transcription of RNA molecules was accomplished using random hexamer primers with a known 20-nt tag sequence at the 5'-end and the RevertAid First Strand H minus cDNA synthesis kit (Thermo Fisher) according to the manufacturer's manual. Second-strand synthesis using Klenow polymerase (Thermo Fisher) was performed, followed by sequence-independent single primer amplification and purification of amplified DNA (PureLink PCR micro kit; Invitrogen) and elution. Total DNA concentration was measured (Qubit fluorometer; Invitrogen) using the dsDNA High-Sensitivity assay. The sequencing library was prepared using the Native Barcoding Kit 96 (SQK-NBD114.96) according to manufacturer's protocol before sequencing on a R10.4.1 flow cell (Oxford Nanopore Technologies).

For metagenomic analysis, the data was uploaded to the cloud platform BugSeq [21], for long-read taxonomic classification.

Screening for *Wolbachia* spp. DNA

Based on the sequences obtained in the metagenomic analysis, specific primer pairs for amplification of *Wolbachia* spp. were designed to facilitate screening for these bacteria by qPCR. Primers and probes were designed with Primer 3 (2.3.7) in Geneious Prime using an assembly of *Wolbachia pipientis* endosymbiont of *Drosophila melanogaster* (OX384529.1).

Primers amplify a 103 bp long fragment in the *hscA* gene (positions 858,261 to 858,363). Forward (GACGATTCAGCACGTAACGC) and reverse primer (TGGAGTAAGAAAGCGCTGCA) were used at a final concentration of 0.4 µM each. The probe (FAM-GCTGGCATAGAAGTTCTTCGC-BHQ1) was used at a final concentration of 0.2 µM.

Results

Aedes albopictus is the dominant Aedes species on Bioko Island

A total of 557 *Aedes* spp. mosquitoes collected from 2020 to 2021 were analysed. The mosquitoes were distributed in 81 pools containing between one and 29 *Aedes* spp. per pool. 71.8% of all collected mosquitoes originated from Malabo, followed by Luba (13.1%), Baney (9.0%) and Riaba (3.6%) (Table 2). Almost every second pool (46.9%) containing 57.8% of all mosquitoes consisted of a mix of *A. albopictus* and *A. aegypti*. 40.7% of total pools with 38.1% of mosquitoes contained *A. albopictus* only and 12.3% of pools with 4.1% of all mosquitoes contained *A. aegypti* only. Most *A. aegypti* positive pools were found in Malabo, followed by Luba. In Baney and Riaba, no *A. aegypti* only positive pool was found. Three mixed *Aedes* spp. pools were found in Baney and one mixed *Aedes* spp. pool in Riaba. These mixed pools consisted of six and five mosquitoes, respectively. In Malabo, 61.2% of the pools were positive for both *Aedes* species.

Table 2: Overview of *Aedes* spp. collected on Bioko Island in 2020 and 2021.

District	Total		*A. aegypti*		*A. albopictus*		*A. aegypti* and *A. albopictus*	
	Pools (n)	*Aedes* spp. (n)	Pools (n)	*A. aegypti* (n)	Pools (n)	*A. albopictus* (n)	Pools (n)	*A. aegypti* and *A. albopictus* (n)
Baney	11	50	0	0	8	44	3	6
Luba	15	73	4	8	6	48	5	17
Malabo	49	400	6	15	14	100	29	294
Riaba	5	20	0	0	5	20	1	5
Total	81	557	10	23	33	212	38	322

Aedes spp. preferably feed on humans

Data on the origin of the last blood meals of the collected mosquitos were generated for 89% (73/81) pools. No traces of a recent blood meal could be detected in 27.4% (20/73) pools, the remaining 72.6% (53/73) pools were positive for a human blood meal as detected by amplification of the *HsRNaseP* gene. In addition to that, three pools (4.1%) were positive for dog DNA and one pool (1.4%) was positive for bird DNA. Sequence analysis identified the bird as chicken (*Gallus gallus*). Out of seven *A. aegypti* positive pools, 57.1% (4/7) were negative for any blood meal, whereas the rest was positive for human DNA (Figure 1). Out of 30 *A. albopictus* positive pools, 33.3% (10/30) were negative for any blood meal, 56.7% (17/30) were positive for human DNA, two pools (6.7%) were positive for human and dog and one pool (3.3%) was positive for human and chicken. 36 pools containing *A. aegypti* and *A. albopictus*, 16.7% (6/36) were negative for any blood meal, 80.6% (29/36) were positive for human DNA and one pool (2.8%) was positive for human and dog DNA.

Wolbachia spp. endosymbiont is highly prevalent

All 33 *A. albopictus* positive pools as well as 97.4% (37/38) of mixed *Aedes* spp. pools were positive for *Wolbachia* spp., while only 20.0% (2/10) of *A. aegypti* positive pools were positive for *Wolbachia* spp. by qPCR (Figure 2A). Cycle quantification (Cq) values of *A. albopictus* and *Wolbachia* spp. were moderately correlated (R^2=0.51) (Figure 2B), while Cq values of *A. aegypti* and *Wolbachia* spp. did not show any correlation (R^2=0.053) (Figure 2C).

Metagenomic sequencing resulted in 17’160 to 175’975 reads per sample. *Wolbachia* spp. were found in nine out of ten samples with a median of 96 reads (range 44 to 269 reads).

No human pathogens found in the tested mosquitoes

All 81 *Aedes* spp. pools tested negative by RT-qPCR for DENV, CHIKV, YFV and ZIKV. In addition, no pool was positive for *Rickettsia* spp.

Discussion

Aedes albopictus is the dominant Aedes species on Bioko Island

A. aegypti was describes as the native Aedes species on Bioko Island and *A. albopictus* was first identified on the island in 2001 [22]. The results of our study strongly indicate that by 2020, *A. albopictus* has emerged as the predominant *Aedes* species. Despite rapid urbanization of the island, the predominant species *A. albopictus* seems to be an all-rounder and was found in all districts, whereas *A. aegypti* was primarily concentrated in the urban area of Malabo.

Aedes spp. preferably feed on humans

The sequencing and qPCR results showed that *A. albopictus* apart from feeding on humans also take blood from domesticated animals including *Gallus gallus domesticus* (chickens) or *Canis lupus familiaris* (dogs). Both alternative blood meal sources were detected in the urban district Malabo where these animals are kept outside and are prone to mosquito bites.

The findings are less clear for *A. aegypti,* as one of the dog sequences originated from a mixed pool consisting *A. albopictus* and *A. aegypti* mosquitoes. The results suggest that *A. aegypti* exhibits a preference for humans as a blood meal source and tends to be more prevalent in densely populated urban areas than in rural regions.

Wolbachia spp. endosymbiont is highly prevalent

Metagenomic analyses were performed on nucleic acids extracted from nine mosquito pools. The abundance of DNA of the endosymbiont Wolbachia enabled us to develop a novel qPCR assay. We tested 65 pools with this qPCR assay and 87% of all samples were found positive for *Wolbachia* spp.

These endosymbionts might play an important role in controlling the spread of vector borne diseases as they alter the metabolism of the mosquito vector resulting in reduced transmissibility of human pathogens. *A. albopictus* mosquitoes are significantly more infected than *A. aegypti* what matches the findings of previous studies e.g. in Malaysia and Panama [23, 24].

No human pathogens found in the tested mosquitoes

Surveillance studies assessing the prevalence of pathogenic Flaviviruses, such as DENV, CHIKV, YFV and ZIKV, and the gram-negative bacterial pathogen *Rickettsia* spp., have not been conducted on Bioko Island. Despite the absence of nucleic acids of these pathogens in the current study, it is important to note that this does not conclusively proof the absence of these viruses on the island. To make a well-founded determination regarding the presence of these viruses in a specific area, comprehensive surveillance data from hospitals and increased screening of mosquitoes are imperative. This data does not exist yet as many Flavivirus infections leading to fever are misdiagnosed as malaria [12] and we expect our sample size to be too small to proof the absence of human pathogens in this region.

Conclusion

Bioko Island has made significant investments in malaria control, focusing on monitoring main malaria vectors belonging to *Anopheles* spp. However, this study highlights the necessity of expanding these control efforts to include other insect vectors, specifically *Aedes* spp. The species composition on the island has undergone a drastic shift in the last two decades. By incorporating advanced ONT sequencing technology, we can now dive deeper into the effects of naturally occurring Wolbachia strains and their vector interaction. This knowledge can be instrumental in implementing artificial Wolbachia infections in mosquitoes as part of an effective vector control strategy.

Abbreviations

YFV: Yellow Fever

DENV: Dengue virus

CHIKV: Chikungunya virus

ZIKV: Zika virus

WHO: World Health Organisation

HLC: Human Landing Catch

CDC-LT: Centre for Disease Control Light Trap

qPCR: quantitative polymerase chain reaction

RT-qPCR: Reverse Transcriptase qPCR

HsRNaseP: human RNaseP

Cq : Cycle quantification

Declarations

Acknowledgements

The authors would like to thank all involved persons from Medical Care Development Global Health for their help in sampling.

Funding

This work was funded through the collaboration of the Swiss Tropical and Public Health Institute and MCD Global Health in Equatorial Guinea as part of the Bioko Island Malaria Elimination Project.

Authors' contributions

TS, SH and JNG conceived the study together with NGvP for her thesis. NRB, GF, RNNO, MLH, PBEE, VONM, GAG, WPP and MB carried out the sampling, performed morphological identification and sample preparation for shipment. NGvP performed molecular analysis under supervision of SH and JNG. NGvP and SH wrote the first draft of the manuscript, which was then completed by JNG. CD reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Supplementary information

References

1. Egid BR, Coulibaly M, Dadzie SK, Kamgang B, McCall PJ, Sedda L, et al. Review of the ecology and behaviour of *Aedes aegypti* and *Aedes albopictus* in Western Africa and implications for vector control. Curr Res Parasitol Vector Borne Dis. 2022;2:100074; doi: 10.1016/j.crpvbd.2021.100074. https://www.ncbi.nlm.nih.gov/pubmed/35726222.

2. Hernandez-Valencia JC, Munoz-Laiton P, Gomez GF, Correa MM. A Systematic Review on the Viruses of Anopheles Mosquitoes: The Potential Importance for Public Health. Trop Med Infect Dis. 2023;8 10; doi: 10.3390/tropicalmed8100459. https://www.ncbi.nlm.nih.gov/pubmed/37888587.
3. Conway MJ, Colpitts TM, Fikrig E. Role of the Vector in Arbovirus Transmission. Annu Rev Virol. 2014;1 1:71-88; doi: 10.1146/annurev-virology-031413-085513. https://www.ncbi.nlm.nih.gov/pubmed/26958715.
4. Paupy C, Delatte H, Bagny L, Corbel V, Fontenille D. *Aedes albopictus*, an arbovirus vector: from the darkness to the light. Microbes Infect. 2009;11 14-15:1177-85; doi: 10.1016/j.micinf.2009.05.005. https://www.ncbi.nlm.nih.gov/pubmed/19450706.
5. Balakrishnan VS. WHO launches global initiative for arboviral diseases. Lancet Microbe. 2022;3 6:e407; doi: 10.1016/S2666-5247(22)00130-6. https://www.ncbi.nlm.nih.gov/pubmed/35659901.
6. Agboli E, Zahouli JBZ, Badolo A, Jost H. Mosquito-Associated Viruses and Their Related Mosquitoes in West Africa. Viruses. 2021;13 5; doi: 10.3390/v13050891. https://www.ncbi.nlm.nih.gov/pubmed/34065928.
7. Poungou N, Sevidzem SL, Koumba AA, Koumba CRZ, Mbehang P, Onanga R, et al. Mosquito-Borne Arboviruses Occurrence and Distribution in the Last Three Decades in Central Africa: A Systematic Literature Review. Microorganisms. 2023;12 1; doi: 10.3390/microorganisms12010004. https://www.ncbi.nlm.nih.gov/pubmed/38276174.
8. Badolo A, Sombie A, Yameogo F, Wangrawa DW, Sanon A, Pignatelli PM, et al. First comprehensive analysis of *Aedes aegypti* bionomics during an arbovirus outbreak in west Africa: Dengue in Ouagadougou, Burkina Faso, 2016-2017. PLoS Negl Trop Dis. 2022;16 7:e0010059; doi: 10.1371/journal.pntd.0010059. https://www.ncbi.nlm.nih.gov/pubmed/35793379.
9. Fofana D, Beugré JMV, Yao-Acapovi GL, Lendzele SS. Risk of Dengue Transmission in Cocody (Abidjan, Ivory Coast). J Parasitol Res. 2019;2019:4914137; doi: 10.1155/2019/4914137.
10. Simo Tchetgna H, Sem Ouilibona R, Nkili-Meyong AA, Caron M, Labouba I, Selekon B, et al. Viral Exploration of Negative Acute Febrile Cases Observed during Chikungunya Outbreaks in Gabon. Intervirology. 2018;61 4:174-84; doi: 10.1159/000495136.
11. Dariano DF, Taitt CR, Jacobsen KH, Bangura U, Bockarie AS, Bockarie MJ, et al. Surveillance of Vector-Borne Infections (Chikungunya, Dengue, and Malaria) in Bo, Sierra Leone, 2012-2013. Am J Trop Med Hyg. 2017;97 4:1151-4; doi: 10.4269/ajtmh.16-0798.
12. Anjorin ET, Olulaja ON, Osoba ME, Oyadiran OT, Ogunsanya AO, Akinade ON, et al. Overtreatment of malaria in the Nigerian healthcare setting: prescription practice, rationale and consequences. Pan Afr Med J. 2023;45:111; doi: 10.11604/pamj.2023.45.111.31780.
13. Agha SB, Tchouassi DP. Urbanization of Aedes mosquito populations and evolution of arboviral disease risk in Africa. Curr Opin Insect Sci. 2022;54:100988; doi: 10.1016/j.cois.2022.100988.
14. Lwande OW, Obanda V, Lindström A, Ahlm C, Evander M, Näslund J, et al. Globe-Trotting *Aedes aegypti* and *Aedes albopictus*: Risk Factors for Arbovirus Pandemics. Vector Borne Zoonotic Dis. 2020;20 2:71-81; doi: 10.1089/vbz.2019.2486.

15. Rogers DJ, Randolph SE. Studying the global distribution of infectious diseases using GIS and RS. Nat Rev Microbiol. 2003;1 3:231-7; doi: 10.1038/nrmicro776.
16. Pietri JE, DeBruhl H, Sullivan W. The rich somatic life of Wolbachia. Microbiologyopen. 2016;5 6:923-36; doi: 10.1002/mbo3.390.
17. Porter J, Sullivan W. The cellular lives of Wolbachia. Nat Rev Microbiol. 2023;21 11:750-66; doi: 10.1038/s41579-023-00918-x.
18. Rasgon JL, Styer LM, Scott TW. Wolbachia-induced mortality as a mechanism to modulate pathogen transmission by vector arthropods. J Med Entomol. 2003;40 2:125-32; doi: 10.1603/0022-2585-40.2.125.
19. Bradley J, Monti F, Rehman AM, Schwabe C, Vargas D, Garcia G, et al. Infection importation: a key challenge to malaria elimination on Bioko Island, Equatorial Guinea. Malar J. 2015;14:46; doi: 10.1186/s12936-015-0579-5. https://www.ncbi.nlm.nih.gov/pubmed/25651929.
20. Akello JO, Leib SL, Engler O, Beuret C. Evaluation of Viral RNA Recovery Methods in Vectors by Metagenomic Sequencing. Viruses. 2020;12 5; doi: 10.3390/v12050562. https://www.ncbi.nlm.nih.gov/pubmed/32438629.
21. Fan J, Huang S, Chorlton SD. BugSeq: a highly accurate cloud platform for long-read metagenomic analyses. BMC Bioinformatics. 2021;22 1:160; doi: 10.1186/s12859-021-04089-5.
22. Toto JC, Abaga S, Carnevale P, Simard F. First report of the oriental mosquito *Aedes albopictus* on the West African island of Bioko, Equatorial Guinea. Med Vet Entomol. 2003;17 3:343-6; doi: 10.1046/j.1365-2915.2003.00447.x. https://www.ncbi.nlm.nih.gov/pubmed/12941021.
23. Bennett KL, Gómez-Martínez C, Chin Y, Saltonstall K, McMillan WO, Rovira JR, et al. Dynamics and diversity of bacteria associated with the disease vectors *Aedes aegypti* and *Aedes albopictus*. Sci Rep. 2019;9 1:12160; doi: 10.1038/s41598-019-48414-8.
24. Ahmad NA, Vythilingam I, Lim YAL, Zabari N, Lee HL. Detection of Wolbachia in *Aedes albopictus* and Their Effects on Chikungunya Virus. Am J Trop Med Hyg. 2017;96 1:148-56; doi: 10.4269/ajtmh.16-0516.
25. Fauver JR, Akter S, Morales AIO, Black WCt, Rodriguez AD, Stenglein MD, et al. A reverse-transcription/RNase H based protocol for depletion of mosquito ribosomal RNA facilitates viral intrahost evolution analysis, transcriptomics and pathogen discovery. Virology. 2019;528:181-97; doi: 10.1016/j.virol.2018.12.020. https://www.ncbi.nlm.nih.gov/pubmed/30616207.
26. Kamau E, Alemayehu S, Feghali KC, Saunders D, Ockenhouse CF. Multiplex qPCR for detection and absolute quantification of malaria. PLoS One. 2013;8 8:e71539; doi: 10.1371/journal.pone.0071539. https://www.ncbi.nlm.nih.gov/pubmed/24009663.
27. Kothera L, Byrd B, Savage HM. Duplex Real-Time PCR Assay Distinguishes *Aedes aegypti* From Ae. albopictus (Diptera: Culicidae) Using DNA From Sonicated First-Instar Larvae. J Med Entomol. 2017;54 6:1567-72; doi: 10.1093/jme/tjx125. https://www.ncbi.nlm.nih.gov/pubmed/28981691.
28. Patel P, Landt O, Kaiser M, Faye O, Koppe T, Lass U, et al. Development of one-step quantitative reverse transcription PCR for the rapid detection of flaviviruses. Virology Journal. 2013;10; doi: Artn

58 10.1186/1743-422x-10-58. <Go to ISI>://WOS:000317654900001.

29. Johnson BW, Russell BJ, Lanciotti RS. Serotype-specific detection of dengue viruses in a fourplex real-time reverse transcriptase PCR assay. J Clin Microbiol. 2005;43 10:4977-83; doi: 10.1128/JCM.43.10.4977-4983.2005. https://www.ncbi.nlm.nih.gov/pubmed/16207951.
30. Broeders S, Garlant L, Fraiture MA, Vandermassen E, Suin V, Vanhomwegen J, et al. A new multiplex RT-qPCR method for the simultaneous detection and discrimination of Zika and chikungunya viruses. Int J Infect Dis. 2020;92:160-70; doi: 10.1016/j.ijid.2019.12.028. https://www.ncbi.nlm.nih.gov/pubmed/31884173.
31. Mantel N, Aguirre M, Gulia S, Girerd-Chambaz Y, Colombani S, Moste C, et al. Standardized quantitative RT-PCR assays for quantitation of yellow fever and chimeric yellow fever-dengue vaccines. J Virol Methods. 2008;151 1:40-6; doi: 10.1016/j.jviromet.2008.03.026. https://www.ncbi.nlm.nih.gov/pubmed/18501437.
32. Socolovschi C, Mediannikov O, Sokhna C, Tall A, Diatta G, Bassene H, et al. Rickettsia felis-associated uneruptive fever, Senegal. Emerg Infect Dis. 2010;16 7:1140-2; doi: 10.3201/eid1607.100070. https://www.ncbi.nlm.nih.gov/pubmed/20587190.
33. Woods ME, Montenieri JA, Eisen RJ, Zeidner NS, Borchert JN, Laudisoit A, et al. Identification of flea blood meals using multiplexed real-time polymerase chain reaction targeting mitochondrial gene fragments. Am J Trop Med Hyg. 2009;80 6:998-1003.
34. Keven JB, Artzberger G, Gillies ML, Mbewe RB, Walker ED. Probe-based multiplex qPCR identifies blood-meal hosts in Anopheles mosquitoes from Papua New Guinea. Parasit Vectors. 2020;13 1:111; doi: 10.1186/s13071-020-3986-6. https://www.ncbi.nlm.nih.gov/pubmed/32111232.
35. Goethert HK, Mather TN, Buchthal J, Telford SR, 3rd. Retrotransposon-Based Blood Meal Analysis of Nymphal Deer Ticks Demonstrates Spatiotemporal Diversity of Borrelia burgdorferi and Babesia microti Reservoirs. Appl Environ Microbiol. 2021;87 2; doi: 10.1128/AEM.02370-20. https://www.ncbi.nlm.nih.gov/pubmed/33158895.
36. MR VDS, Gu X, Ward MP, Kirkland PD. Development and evaluation of real-time PCR assays for bloodmeal identification in Culicoides midges. Med Vet Entomol. 2016;30 2:155-65; doi: 10.1111/mve.12163. https://www.ncbi.nlm.nih.gov/pubmed/26854008.

Figures

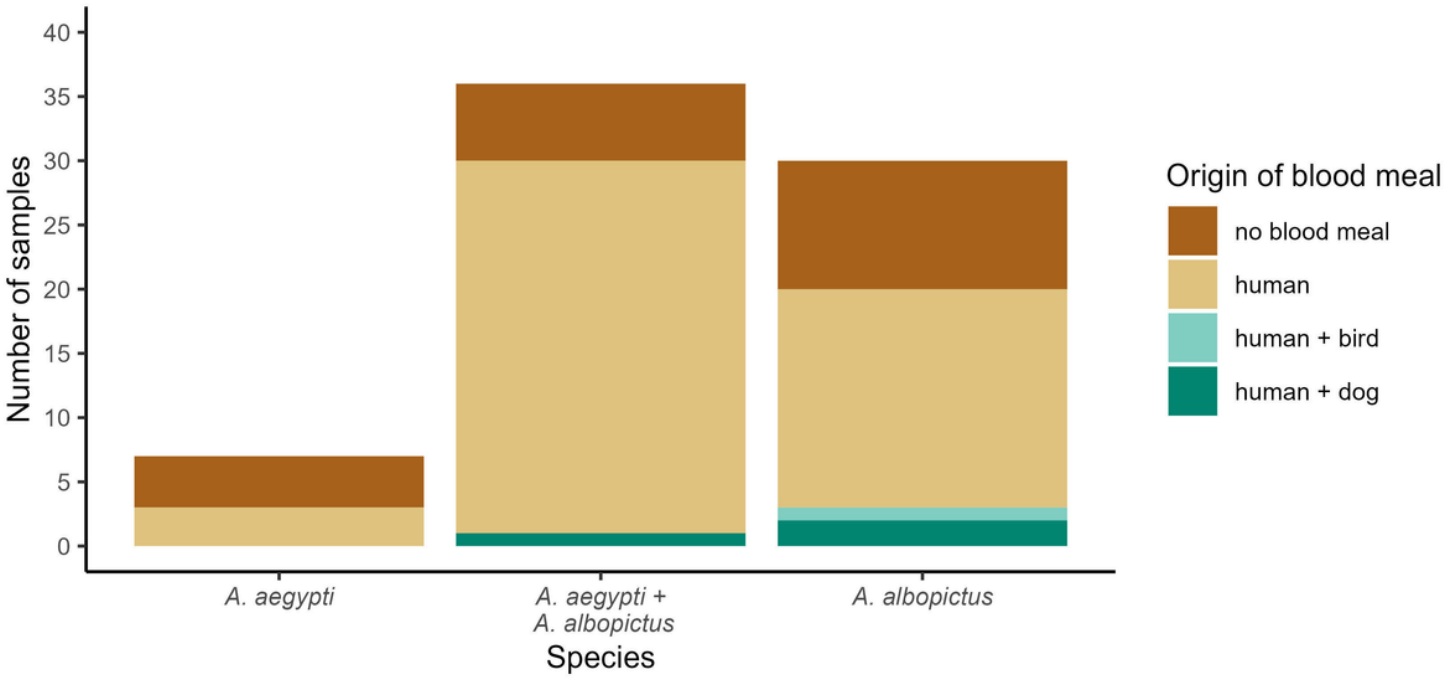

Figure 1

The last blood meal origin of the 73 Aedes spp. pools is split according to the Aedes spp. identified. Human DNA (HsRNaseP) was present in 57.1 % of the A. aegypti pools and 56.7% of the A. albopictus pools. Additionally, 6.7% of A. albopictus pools have feed on dogs as well. The mixed pools had an 80.6% positivity rate for human DNA and 2.8% were positive for human and dog DNA.

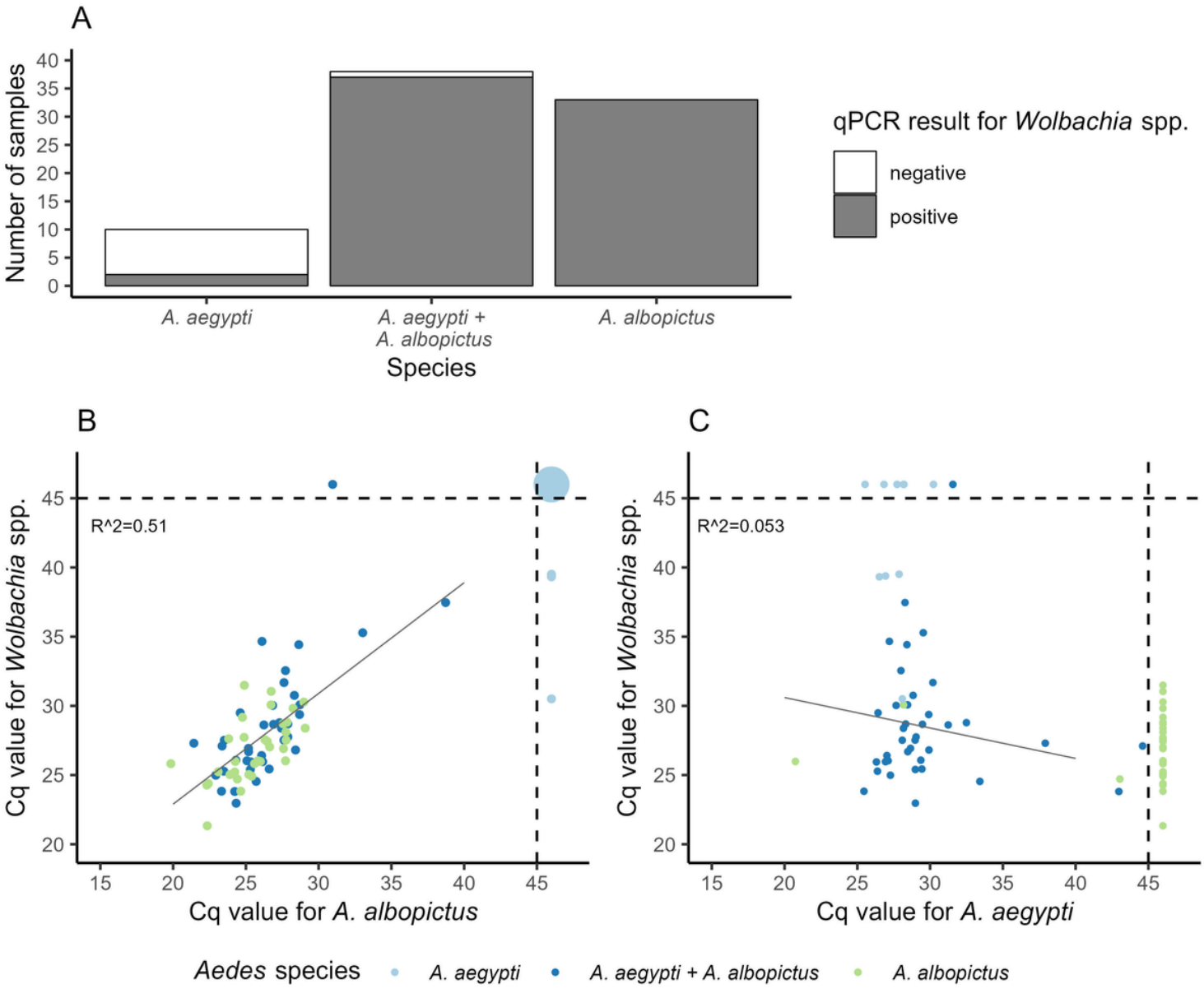

Figure 2

Detection of *Wolbachia* spp. in different *Aedes* spp. A. Number of *Wolbachia* spp. positive samples for *A. aegypti*, *A. albopictus* and mixed species pools stratified according to detection of *Wolbachia* spp. B. Cq values for *A. albopictus* (x-axis) and *Wolbachia* spp. (y-axis) with linear regression line. C. Cycle quantification (Cq) values for *A. aegypti* (x-axis) and *Wolbachia* spp. (y-axis) with linear regression line. Negative values are attributed a Cq value of 46 for visualization. Circle size corresponds to the number of sample with this Cq value.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfile1AedespopulationBioko.docx