RESEARCH ARTICLE

Entomological indicators of malaria transmission and insecticide resistance profile of *Anopheles gambiae* at the early phase of irrigated rice farming in the forest area of central Cameroon [version 1; peer review: 1 approved with reservations, 1 not approved]

Emmanuel Elanga-Ndille¹, Achille Binyang¹,², Cyrille Ndo³,⁴, Tatiane Assatse¹,², Lynda Nouage¹,², Magellan Tchouakui¹,², Billy Tene-Fossog¹, Sevilor Kekeunou², Charles S. Wondji¹,⁵

¹Department of Medica Entomology, Centre for Research in Infectious Diseases (CRID), Yaoundé, P.O.Box: 13591, Cameroon
²Department of Animal Biology and Physiology, Faculty of Science, University of Yaoundé 1, Yaoundé, P.O.Box: 812, Cameroon
³Department of Biological Sciences, Faculty of Medicine and Pharmaceutical Sciences, University of Douala, Douala, Cameroon
⁴Department of Parasitology, Centre of Research in Infectious Diseases (CRID), Yaoundé, P.O.box: 13591, Cameroon
⁵Vector Biology, Liverpool School of Tropical Medicine, Liverpool, L35QA, UK

Abstract

**Background:** Cameroonian authorities have recently established irrigated rice projects across the country. The assessment of the impact of such projects in the transmission of vector-borne diseases, such as malaria, imposes to compare the situation before and after their implementation in a given locality. In Bankeng, a village in a forest area in Cameroon, no preliminary data on malaria transmission indicators was collected before the implantation of irrigated rice farming. To attempt to catch up for this shortcoming 6 months after the first rice seedlings were sown., the present study aimed to generate data which could serve as a baseline to assess the impact of irrigated rice farming in Bankeng.

**Methods:** A cross-sectional study was performed at the end of the rainy season in July 2018. Mosquitoes were sampled by night collections on human volunteers, identified morphologically and members of different complexes further sorted to species using molecular tools. *Plasmodium* infectious status was determined by Taqman genotyping. To assess resistance profile to insecticides, WHO bioassays were performed using two-to-five days old females *An. gambiae* s.l. reared from larval collections in Bankeng. Furthermore, the molecular basis of resistance were investigated

Open Peer Review

**Approval Status**

1. Armel Djenontin, Université d'Abomey-Calavi/Centre de Recherche Entomologique de Cotonou (CREC), Cotonou, Benin
2. Judicaël Obame-Nkoghe, Université des Sciences et Techniques de Masuku (USTM), Franceville, Gabon

Any reports and responses or comments on the article can be found at the end of the article.
Results: *An. gambiae* s.l represented 98% of the 1087 mosquitoes collected with *Anopheles gambiae* as the predominant species. The total human biting rate was 44.5 bites/person/night. Entomological inoculation rate was 3.8 ib/p/n. The Bankeng *An. gambiae* population exhibited a high level of resistance to almost all insecticides except to organophosphates with a high frequency of L1014F kdr mutation (93.9%) and a 6-fold over-expression of CYP6P3 P450 gene.

Conclusion: In the absence of preliminary data before the implementation of the irrigated rice fields; the present study provides interesting data which could help for the future assessment of the impact of irrigated rice cultivation on malaria transmission in the locality of Bankeng.

Keywords
Malaria, risk of transmission, irrigated rice, Bankeng, An. gambiae, insecticide resistance.
Introduction
According to the United Nations projections, the human population in sub-Saharan African countries could double from 1.2 billion to 2.5 billion by 2050\textsuperscript{1,2}. One of the consequences of such significant population growth is the need to increase food production in order to sustain the population. To cope with the pressure of feeding this growing population, numerous African governments have decided to improve and expand their agriculture production. Thus, in the past decades, dramatic transformations of agricultural practices have been observed across the continent, including intensive irrigation farming and use of pesticides and chemicals, with one notable example being an expansion in irrigated rice farming\textsuperscript{3,4}. In response to the international food crisis of 2008, many African countries have embarked on ambitious programs to boost their rice production capacity\textsuperscript{5}. For this purpose, rice has become the most rapidly growing food resource with irrigated paddies production increasing by 9.5% in the last 10 years\textsuperscript{6}.

Numerous studies across the continent reported that irrigated rice cultivation is associated with the creation of permanent larval habitats that support higher densities of malaria vectors and sometimes malaria transmission\textsuperscript{7,20}. For example, a study in Central Kenya reported higher densities of *An. arabiensis* mosquitoes in irrigated rice agro-ecosystem compared to non-irrigated one\textsuperscript{8,11}. Such observation was also reported in villages with rice irrigation in Tanzania\textsuperscript{12}. In the same way, human-biting rate of malaria vectors was reported 10-fold higher in rice growing areas compared to those without rice cultivation in Burkina-Faso\textsuperscript{9}. One other study in Madagascar showed that irrigated rice fields provide more than 90% of potential habitats for *An. funestus* mosquito species\textsuperscript{14}.

However, although it is reported that irrigated farming is associated with an increase in the prevalence of malaria, numerous studies reported a more complex picture of the effect of irrigation on malaria transmission risk. Indeed, contrasting results are reported on the effect of irrigation on entomological parameters related to malaria transmission risk\textsuperscript{13,22}. For example, significantly higher biting rates and an increase in malaria transmission has been reported in an irrigated rice ecosystem in several African countries such as Madagascar\textsuperscript{14}, Ethiopia\textsuperscript{16,7}, Kenya\textsuperscript{18}, Cameroon\textsuperscript{19}, Tanzania\textsuperscript{20} and Côte d’Ivoire\textsuperscript{21,22}. In contrast, findings from other studies reported that irrigated agriculture did not influence malaria transmission risk\textsuperscript{23,24}. This contrast highlights the need to assess the impact of the increasing development of irrigated agricultural projects such as in Cameroon notably at the local level for the implementation of efficient strategies to better protect the population against malaria.

The present study was conducted with the purpose of estimating entomological indicators of malaria transmission and investigating the insecticide profile of *Anopheles* mosquitoes at the early phase of irrigated rice farming in the locality of Bankeng, situated in the forest central Cameroon. To the best of our knowledge, no preliminary study has been carried-out to generate baseline data on malaria transmission profile in this locality before the implementation of the irrigated rice fields. This study attempted to catch up for this shortcoming 6 months after the first rice seedlings were sown, by providing data which could serve as baseline to assess the long term impact of irrigated rice farming in Bankeng.

Methods
Study area
A cross-sectional study was carried out in the village of Bankeng (4° 38’ 43” N; 12° 13’ 03” E) in the district of Nkoteng, located 92 km from Yaoundé along the forest area of Central Cameroon (Figure 1). Early in 2018, 20 hectares of irrigated rice fields were established as part of a governmental effort to minimize rice importation into the country, while aiming to produce about 310 000 tonnes of rice per annum. The rural area has an equatorial climate with high humidity characterized by two rainy seasons, extending from March to June and September to November with rainfall averaging 1,000–1,500 mm each year. The village has a population of 250 inhabitants who are mainly subsistence farmers cultivating maize, cassava, yams, groundnut and vegetables. Furthermore, some villagers work in the watermelon fields located 3km away from the village. The area is located along the Sanaga River, which is used to irrigate the rice fields.

Mosquito collection
Mosquitoes were collected at the end of the rainy season in July 2018 during the first rice harvest, 6 months after the first seeding. Adult female mosquitoes were collected using the human landing catches (HLC) performed from 06:00 PM to 06:00 AM inside and outside of human sleeping rooms by volunteers recruited in the village (four outdoors and four indoors). All volunteers gave free and informed consent to participate in the study. Collections were done during three consecutive nights in eight randomly selected sites for capturing mosquitoes. In each house, two collectors were positioned, one inside the house and the other outside, to collect mosquitoes. For each 2 hour of collection, collectors collected mosquitoes for 1h55 minutes and rested for 5 minutes, during which they exchanged positions. During the time of collection, the collectors sat quietly on a small chair and exposed part of their legs (up to the knees) and arms; when they felt landing mosquitoes, they turned on a torch and collected the mosquitoes by inverting a small glass tube over it. The entry of the tube was blocked by a cotton and the tube was transferred into labeled bags assigned for each collection time. Collected Anopheline mosquitoes were subsequently identified under a stereomicroscope, counted by species according to the morphological identification keys of Gillies and De Meillon\textsuperscript{25} and Gillies and Coetzee\textsuperscript{26}, and time of collection, and preserved in 1.5 ml Eppendorf tubes with a hole pierced in it and kept in a plastic container containing silica gel. Samples were transported to the Centre for Research in Infectious Diseases (CRID) and kept in –20°C until molecular analyses.

PCR-species identification
Genomic DNA, extracted using Livak protocol\textsuperscript{27} from the legs, wings and abdomen of a sub-sample of 150 individuals randomly selected from field-collected adult mosquitoes, was used to perform the short interspersed elements (SINE) PCR to differentiate members of the *An. gambiae s.l.* complex following...
the protocol of Santolamazza et al. using F6.1a (TCGCCCTTA GACCTTGCGTTA) and R6.1b (CGCTTCAAGAATTCGAGATAC). PCR was performed in Gene Touch thermal cycler (Model TC-E-48DA). Briefly, PCR was carried out using 0.34 mM of each primer and 1 μl of genomic DNA as template in 15 μl reactions containing 10X Kapa Taq buffer A (KB 1003), 0.2 mM dNTPs (DM-516404), 1.25 mM MgCl₂ (KB 1001), 0.04U Kapa Taq (Kapa biosystems 5U/μl, Cat: 07958471001). Reaction conditions used were 95°C for 5 minutes; followed by 35 cycles of 95°C for 30 seconds, 54°C for 30 seconds; 72°C for 1 minute; and a final extension at 72°C for 10 minutes. PCR products were separated on 2% agarose gel by electrophoresis.

Estimation of *Plasmodium* Infection rate

The sporozoite infection rate was estimated using the TaqMan assay according to the protocol described by Bass et al. with genomic DNA extracted from the head and thorax only from field-collected female mosquitoes. Samples were run on a Stratagene Mx3000P™ (Agilent, Santa Clara, CA, USA). Briefly, 1 μl of DNA sample was used as template in a 3-step PCR program with a pre-denaturation at 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The primers (Falcip+::TCT-GAA-TACGAGTGT-GTG, OVM+::CTG-AAT-ACA-ATAGGCC, Plas F: GCCTAG-TTA-CTGAATA-AGA-TGA-GCT-TG, Plas r: GAA-AAT-AGA-ATT-TCA-CCTCTG-ACA) were used together with two probes labeled with fluorophores: FAM to detect *Plasmodium falciparum*, and HEX to detect *Plasmodium ovale*, *Plasmodium vivax* and *Plasmodium malariae* alongside the positive controls.

Insecticide susceptibility assays

Insecticide susceptibility tests were carried out using 2–5 days old F₀ female mosquito from field-collected larvae following the WHO protocol. Insecticide impregnated papers were supplied by WHO reference center (Vector Control Research Unit, University Sains Malaysia, Penang, Malaysia). Mosquitoes were tested using discriminating doses of eight insecticides: the pyrethroids deltamethrin (0.05%) and permethrin (0.75%), the pseudo-pyrethroid etofenprox (0.05%), the organochlorine DDT (4%), the organophosphates fenithrothion (1%) and the carbamate bendiocarb (0.1%) and propoxur (0.1%). In addition, synergist assays with 4% piperonyl butoxide (PBO) and diethyl maleate (DEM) impregnated papers were carried out to investigate the role of cytochrome P450s and glutathione S-transferases respectively in the resistance exhibited by the *Anopheles* mosquitoes in Bankeng. Approximately 20–25 mosquitoes per tubes with 4 replicates were exposed to insecticide impregnated papers for 1h. Meanwhile, two batches of 20 mosquitoes exposed to untreated paper was used as a control. After the exposure period, mosquitoes were transferred to a clean holding tube and the mortality was recorded 24 hours after. Resistance status was evaluated according to the WHO criteria which classifies mortality rates less than 90% as indicative of resistance and those greater than 98% as
indicative of susceptibility. Mortality rates between 90–98% suggest a probable resistance that needs to be confirmed. The mosquitoes alive after exposure were kept in -80°C and the dead individuals were stored in silica gel and kept in -20°C.

Genotyping of knockdown resistance (kdr) mutations
Total DNA extracted from field-collected adult mosquitoes that were randomly selected was used for the detection of L1014F kdr mutations conferring resistance to pyrethroids and DDT in An. gambiae s.l species. The kdr 1014 mutation alleles were genotyped following a PCR protocol as previously described using positive and negative controls. Briefly, PCR was performed with a reaction volume of 10 μl containing 1 μl of genomic DNA, 5μl of SensisMix DNA kit (catalog: SM2-717104), 0.125μl of the L1014F-kdrw probe and 3.875 μl of sigma water. Samples were run on a MX3000P™ Multiplex quantitative PCR system (Stratagene) using the temperature cycling conditions of: 10 minutes at 95°C followed by 40 cycles of 95°C for 10 seconds and 60°C for 45 seconds.

Transcriptional profiling of two major resistance genes in An. gambiae
We assessed the expression profile of two cytochrome-dependent monoxygenases: CYP6M2 and CYP6P3, which have previously been reported as the main drivers of metabolic resistance to pyrethroid and DDT in An. gambiae and An. coluzzii in Cameroon. This analysis was performed by quantitative real time (qRT)- polymerase reaction chain (PCR) following a protocol previously described. RNA was extracted from three biological replicates from Bankeng female mosquitoes resistant (R) (alive after 24 hr exposure to deltamethrin), control (C) (not exposed to any insecticide) and susceptible Kisumu mosquitoes (S). The extraction was performed using the RNaseasy Mini Kit (Qiagen, Germany; Cat Number: 74104) according to the manufacturer’s protocol. 1 μg total RNA from each of the three biological replicates was used as the template for cDNA synthesis using Superscript III (Invitrogen, Carlsbad, CA, US; Catalog number: 18080093), according to the manufacturer’s instructions. The qPCR assays were carried out in a MX 3005 real-time PCR system (Agilent, Santa Clara, CA, USA) using Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent, catalog number: 600882). A total of 10 ng of cDNA from each sample was used as template in a three-step program involving a denaturation at 95°C for 3 min followed by 40 cycles of 10 s at 95°C and 10 s at 60°C and a last step of 1 min at 95°C, 30 s at 55°C and 30 s at 95°C. The relative expression and fold-change of each target gene in R and C relative to S was calculated according to the 2−ΔΔCT method incorporating PCR efficiency after normalization with two housekeeping RSP7 (ribosomal protein S7 AGAP010592) and GADPH (vectorBase ID:AGAP009945). For this analysis the following primers were used: for RPS7: forward (5′-ATT-GCCGAGCCGGCATTCT-3′) and reverse (5′-GACGCCGA-TACGCTTGGCGGA-3′) primers, for CYP6M2: forward (5′-TGAGTGTGACGTTCCGGC-3′) and reverse (5′-TCGTGTTCGTCGCACCGGT-3′), for CYP6P3 forward (5′-ATAGTCCACAGACGGTACCGGAG-3′) and reverse (5′-ATAGTCCACAGACGGTACCGGAG-3′) and for GADPH:

Table 1. Daily human biting rate (ma), sporozoite infection rate and entomological inoculation rate (EIR) of Anopheles gambiae collected indoor and outdoor in Bankeng, central Cameroon, in July 2018.

Data analysis
To estimate the level of malaria transmission in Bankeng during the sampling period, the following entomological indicators were considered: i) The biting rate (m.a = number of bites per person per night or b/p/n), calculated as the number of mosquitoes collected per night divided by the number of collectors; ii) The infection rate, estimated as the proportion of Plasmodium infected mosquitoes among the total number analyzed by Taqman PCR; iii) The entomological inoculation rate (EIR) defined as the number of infected bites per person per night (ib/p/n) and calculated as the product of man biting rate and the Plasmodium infection. The biting activity of malaria vectors in the study site was also assessed by monitoring mosquito aggressiveness during each night of collection. This was assessed by estimating the mean number of mosquitoes landing per person per 2 hours. The chi-square Pearson test was used to compare both proportions of outdoor and indoor collected mosquitoes, as well as proportions of Plasmodium infected mosquitoes inside and outside of habitats. Regarding the insecticide susceptibility tests, for each insecticide, the mortality rate was estimated as the proportion of dead mosquitoes among the total number tested. All graph and data analyses were conducted using GraphPad Prism v 7.00.

Results
Mosquito collection and Anopheline biting rate
A total of 1087 female mosquitoes were collected during 24 person-nights. Morphological identification of the caught mosquitoes revealed a predominance of Anopheles gambiae s.l. (98%) with Culex spp, Mansonia spp and Aedes spp also detected. Molecular analysis of 200 specimen of An. gambiae s.l. randomly selected, revealed that 98.5% (198/200) were An. gambiae, while only 2 mosquitoes were identified as An. coluzzii.

An overall biting rate of 44.5 bites/person/night (b/p/n) was recorded for An. gambiae s.l. during the study period. With a biting rate of 50.2 b/p/n outdoor collections and of 38.9 b/p/n indoor collections, these malaria vectors appeared to be more exophagic (χ²=34, df =1, P<0.0001) (Table 1; underlying data). The night human biting cycle (Figure 2; underlying data) showed peak biting activity for An. gambiae between 22:00 and 24:00 indoor and between 24:00 and 02:00 for outdoor. After the peak, the aggressiveness of An. gambiae dropped progressively both indoor and outdoor, but remained higher than 6 b/p/2h until the end of collection at 6:00 am.

![Table 1](https://via.placeholder.com/150)

Table 1. Daily human biting rate (ma), sporozoite infection rate and entomological inoculation rate (EIR) of Anopheles gambiae collected indoor and outdoor in Bankeng, central Cameroon, in July 2018.
Sporozoite infection rates and entomological inoculation rates
The head and thorax from a total of 654/1069 (61.2%) An. gambiae field-collected mosquitoes randomly selected were examined for Plasmodium infection using TaqMan assay. Overall, sporozoites were detected in 8.6% (56/654) of tested mosquitoes with marked difference observed ($\chi^2=15.86$, df =1, P<0.0001) between indoor (12.5%; 45/360) and outdoor (3.7%; 11/294) collections. Overall, P. falciparum was predominant with 82.1% (46/56) of all infections being due to this species alone, whereas 17.9% (10/56) were due to mixed infections by P. falciparum and either P. ovale, P. vivax or P. malariae.

The estimation of the entomological inoculation rate done based on the Anopheles aggressiveness and the mosquito Plasmodium infection rate, led to an EIR of 3.8 infected bites/person/night in the village. However, this entomological indicator was higher indoors (4.9 ib/p/n) than outdoors (1.8 ib/p/n).

Insecticide susceptibility bioassays
A total of 880 F$_1$ An. gambiae mosquito emerged from larvae collected from the field were tested to assess the resistance profile to eight insecticides (Figure 3; underlying data$^a$). A very high level of insecticide resistance was observed with type I and type II pyrethroids with only 5 ± 3.54% and 26.13 ± 5.65% mortality recorded after exposure to permethrin and deltamethrin, respectively. Resistance was also observed for the pseudo-pyrethroid etofenprox with mortality rate of 1.25 ± 1.25%. A high level of resistance was also observed for the organochlorine DDT with a mortality rate of 2.50 ± 1.44%. An. gambiae displayed resistance to the two carbamates tested with mortality rates of 68.42 ± 3.10% for bendiocarb and 57.71 ± 2.99% for propoxur. Concerning the organophosphates, a full susceptibility was observed for fenithrotion.

Synergist assays with PBO revealed a partial recovery of susceptibility for deltamethrin with an increased mortality rate from 26.13 ± 5.65% to 83.61 ± 12.40% and 66.54 ± 13.56% after pre-exposure to PBO and DEM, respectively.

Molecular characterization of resistance mechanisms
The genotyping of the L1014F kdr mutation from 115 field collected mosquitoes randomly selected allowed for the identification of 101 homozygotes (RR) and 14 heterozygotes (RS) resistant mosquitoes with frequency of the resistant allele (L1014F) of 93.9%.

Analysis of the transcription profile of the two candidate resistant genes CYP6M2 and CYP6P3 previously associated with resistance to pyrethroids and DDT in An. gambiae mosquito from Cameroon, showed an over-expression of CYP6P3, while CYP6M2 was not differentially expressed between resistant and susceptible mosquitoes (Figure 4; underlying data$^a$). CYP6P3

Figure 2. Night biting cycle of An. gambiae in the locality of Bankeng, central Cameroon.
Figure 3. Susceptibility profile of *Anopheles gambiae* Bankeng population to insecticides. Recorded mortalities following 60-min exposure to different insecticides is shown as mean ± SEM.

Figure 4. Differential expression of two resistance candidates genes measured by qRT-PCR between *An. gambiae* mosquitoes collected in Bankeng in July 2018 and susceptible laboratory strain Kisumu. (**)=p<0.001.

exhibited a 5.9 fold change in Bankeng resistant mosquitoes when comparing to the Kisumu susceptible lab strain (P<0.0012). Both genes were significantly over-expressed in alive field mosquitoes compared to unexposed (control) ones. On the other hand, when comparing unexposed and susceptible mosquitoes, *CYP6P3* was not differentially expressed between both strains, whereas the *CYP6M2* was under-expressed in unexposed field mosquitoes (P<0.001).

Discussion

Due to the absence of preliminary data on the transmission of vector-borne diseases before the recent establishment of irrigated rice farming in the village of Bankeng, it could be difficult in the long term to assess the impact of this project on the transmission of diseases such as malaria. To attempt to address this shortcoming at the early phase of rice fields implantation, the present study provides data on entomological indicators of
malaria transmission and the insecticide resistance profile of the main vector found in this village. However, since results presented here correspond to one single time frame collection, the absence of replicates represents an important limitation for this study. Nevertheless, given that robust preliminary data do not exist, although generated as part of a cross-sectional survey, results from this study could be used to assess the impact of irrigated rice farming on malaria transmission indicators in Bankeng, by comparing at least, the data from this study with further data collected in the same period.

The morphological identification of field collected mosquitoes revealed that members of *Anopheles gambiae* complex were the sole Anopheline species found in Bankeng during the study period. Furthermore, molecular analyses showed that *An. gambiae* is the predominant species found within the complex in the study area. This result is in line with previous observations showing that *An. gambiae* is the predominant species within the *Anopheles gambiae* complex in rural areas in Central Africa. However, a longitudinal survey would certainly have been more informative of the specific composition of these malaria vectors in the village of Bankeng.

*Anopheles gambiae* biting activity peaked between 10:00 pm and 02:00 am and did not deviate from the habitual pattern commonly observed in the forest area in Central Cameroon and described earlier by Gilles and Meillon. This activity remained high until 6:00 AM suggesting that human population of Bankeng could be bitten by *An. gambiae* mosquito in the early morning, although this assumption was not investigated here since mosquito collection stopped at 6:00 AM. For further studies in the locality, it would be interesting to perform mosquito collection beyond 6:00 AM to have a clearer understanding of the early morning biting rate of local mosquitoes which could further expose the populations to malaria risk. A higher proportion of mosquitoes were collected outdoors than indoors suggesting an exophagic tendency of *Anopheles* mosquito species in Bankeng. This suggests that human population may be more exposed to malaria vectors bites outside than inside their habitation. High values of HBR and proportions of *Plasmodium* infected mosquitoes reveals that malaria transmission intensity would be high in Bankeng during the sampling period. These results corroborate previous studies which commonly reported similar relatively high mosquitoes infection rate during the rainy season in forest areas in southern Cameroon.

Unfortunately, the emergence and spread of resistance in *Anopheles* populations against pyrethroids, the only class of insecticides available for use on LLINs is jeopardizing the effectiveness of this strategy for malaria control. Thus, to guarantee the success of the implementation of a strategy based on the use of LLINs in a locality, it is important to know the resistance profile of the vectors present there. Here, *An. gambiae* mosquito was found to be resistant to multiple insecticides used in public health including pyrethroids. This pattern is similar to what has been commonly reported across Cameroon and confirms that insecticide resistance is widespread in *An. gambiae* mosquito across the country. This high insecticide resistance level, may represent a real threat to the effectiveness of the implementation of standard pyrethroid treated nets to protect inhabitants of Bankeng against malaria.

This threat is as serious as the results of the present study showed that resistance to pyrethroid is driven by high frequency of the kdr mutation and overexpression of at least one P450 genes (the CYP6P3) in the *An. gambiae* populations. This finding revealed that in order to ensure the effectiveness of mass distribution of LLINs in the village of Bankeng, it’s absolutely important not to distribute bed nets treated with only a pyrethroid but with a combination of a pyrethroid and the synergist piperonyl butoxide (PBO). Such PBO-pyrethroid-treated long-lasting insecticidal nets were shown to be more effective than the standard pyrethroid LLINs in reducing malaria infection prevalence in areas with high pyrethroid resistance of primary vectors. Interestingly, this idea is reinforced by the significantly high mortality in *Anopheles gambiae* mosquitoes from Bankeng recorded with synergist bioassays. One other measure that can be used to mitigate insecticide resistance in the *An. gambiae* mosquito population in Bankeng could be mosquito larval control using bacterial larvicides such as, *Bacillus thuringiensis* var. *israelensis* (Bti) and/or *Bacillus sphaericus* (Bs). In fact, these two bacterial larvicides which were reported to be effective in controlling malaria vectors, could be applied in mosquitoes breeding sites formed by irrigated rice fields. This will help in reducing the density of adult mosquitoes that could emerge from these breeding sites. The reduction of mosquito density will lead to the limitation of human-vector contact and consequently, to the reduction of malaria transmission risk.

**Conclusion**

The present study shows that the study site is an area of high malaria transmission intensity. This transmission which mainly occurs inside of habitations is ensured mainly by *An. gambiae* mosquito species which has already developed multiple resistances to almost all insecticides used in public health. These results provide baseline data that could be used for monitoring and evaluation of the long term impact of irrigated rice systems on malaria transmission in the surveyed area. This study also calls on the awareness of national decision makers to implement measures towards improving malaria control strategies such as distribution of PBO-based nets and/or larviciding in a bid to curtail the high risk of malaria transmission in Bankeng. Moreover, policy makers could establish health services in the village to educate the local communities on
personal protection measures and environmental management in order to avoid risk of malaria transmission.

Ethical approval and consent to participate

Ethical clearance was obtained from the National Ethics Committee of Cameroon’s Ministry of Public Health (N°2018/04/1000/CE/CNERSH/SP) in conformity to the WMA Declaration of Helsinki. The study was carefully explained to the chief and inhabitants of Bankeng to obtain their authorization to work in the village. Informed verbal consent was obtained from household owners for using their houses for mosquito collection. Participation in mosquito collection was strictly voluntary and only those adequately trained on the vector collection were allowed to work in the village. Informed verbal consent to the chief and inhabitants of Bankeng to obtain their authorization to work in the village. Research protocols were approved by the study’s Institutional Review Board.

Data availability

Underlying data

Acknowledgments

We thank Dr Micheal KUSIMO and Mr Francis NEMNGO; native speakers of English language for accepting to review and edit this paper.

References
37. Atangana J, Fondjo E, Fomena A, Elanga-Ndille E, Kwiatkowska RM, Platt N, Poupardin R, Antonio-Nkondjio C, Fossog BT, Kopya E, Tene BF, Poupardin R, Costantini C, Martinez-Torres D, Chandre F, Williamson M, Bass C, Nikou D, Blagborough AM, Gillies MT, De Meillon B, Ijumba J, Shenton F, Clarke S, Robert V, Verhave JP, Ponnudurai T, PubMed Abstract | Publisher Full Text

29. Seasonal variations of malaria transmission in Western Cameroon highlands: entomological, parasitological and clinical investigations. Journal of Cell and Animal Biology. 2009; 9(3): 033-038. Reference Source

31. Wondji C, Simard P, Petracca V, et al.: Species and populations of the Anopheles gambiae complex in Cameroon with special emphasis on chromosomal and molecular forms of Anopheles gambiae. s.s. J. Med. Entomol. 2005; 42(6): 998-1005. PubMed Abstract | Publisher Full Text

33. Landscape and ecological divergence between incipient species of the malaria mosquito Anopheles gambiae. PLoS One. 2012; 7(6): e39453. PubMed Abstract | Publisher Full Text | Free Full Text

35. Anthropogenic habitat disturbance and ecological divergence between incipient species of the malaria mosquito Anopheles gambiae s.s. (Diptera: Culicidae) in five agricultural ecosystems from Côte-d’Ivoire. Bull Soc Pathol Exot. 2006; 99(4): 278-282. PubMed Abstract

38. Seasonal prevalence of malaria vectors and entomological inoculation rates in the rubber cultivated area of Niente, South Region of Cameroon. Parasit Vectors. 2012; 5: 197. PubMed Abstract | Publisher Full Text | Free Full Text

40. Menze BD, Wondji MJ, Tchagpa W, et al.: Bionomics and insecticide resistance profiling of malaria vectors at a selected site for experimental hut trials in central Cameroon. Malar J. 2018; 17(1): 317. PubMed Abstract | Publisher Full Text | Free Full Text

42. Elevated Plasmodium falciparum infection rates and high pyrethroid resistance in malaria vectors in a forested area of Cameroon highlight challenges of malaria control. Parasit Vectors. 2018; 11(1): 157. PubMed Abstract | Publisher Full Text | Free Full Text

45. Elevated Plasmodium falciparum infection rates and high pyrethroid resistance in malaria vectors in a forested area of Cameroon highlight challenges of malaria control. Parasit Vectors. 2018; 11(1): 157. PubMed Abstract | Publisher Full Text | Free Full Text

46. Anopheles gambiae s.s. (Diptera: Culicidae) in five agricultural ecosystems from Côte-d’Ivoire. Bull Soc Pathol Exot. 2006; 99(4): 278-282. PubMed Abstract

Wellcome Open Research 2020, 5:190 Last updated: 23 MAR 2022
Open Peer Review

Current Peer Review Status:  ✗  ?

Version 1

Reviewer Report 14 October 2020

https://doi.org/10.21956/wellcomeopenres.17621.r40478

© 2020 Obame-Nkoghe J. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Judicaël Obame-Nkoghe
1 Université des Sciences et Techniques de Masuku (USTM), Franceville, Gabon
2 Centre International de Recherches Médicales de Franceville (CIRMF), Franceville, Gabon

Major revisions:

1. Throughout reading the study, the authors position the study as a response to the lack of preliminary data about indicators of malaria transmission before the implementation of rice cultivation. However, in fact, the authors undertook the study six months after the start of rice crops, especially at the first harvest. This may confuse the understanding of the objectives of the study. This work is scientifically relevant and of high quality, although there is a lack of replicates. However, it does not constitute a factual evaluation prior to the implementation of rice cultivation in Bankeng, but rather an evaluation 6 months after the implementation the start of the crops. The conclusions should take this crucial circumstance into account. I highly recommend to the authors to reposition the orientation and the main objective of this study, or present things more simply. Six months after crop implementation may be sufficient to detect an impact on vector population densities, and therefore on indicators of transmission. However, subsequent evaluations could make it possible to detect differences in the indicators beyond six months after the implementation of the cultures, and thus provide a basis for comparison.

2. Methods, Mosquito collection: It is not clear how volunteers were distributed for mosquito sampling. The authors stated that volunteers were ‘recruited in the village (four outdoors and four indoors)’. Based on this cote, readers are led to understand that there should be 8 volunteers who are doing the sampling. However, they don't explain how many volunteers are simultaneously capturing mosquitoes during a set of 2 hours of collection, before turning over from inside to outside of the rooms, or from outside to inside. The authors should also declare the number of sleeping rooms simultaneously exploited during a set of 2 hours of collection. The sampling has been conducted from 6PM to 6AM (12 hours). Without all these details, readers could be confused, and it may not be easy to reproduce the sampling following the design used.

3. Methods: The authors stated that F0 female mosquitos from field-collected larvae. The
authors did not describe how larvae were collected and how larvae were reared until adults of 2-5 days. Please, add these steps, as these are important for those who may want to reproduce this study.

4. Results: The authors made a subset of 200 mosquitoes selected randomly for molecular identification. However, they did not justify the necessity and the significance of making the molecular identification on a subset of collected mosquitoes, although the selection was made randomly. They must justify it, in order to make the procedure more understandable, [surtout] they presented the molecular method of identification as a major procedure for the study. In the case for example, references already exist to justify the subsetting, they must mention that, otherwise this may constitute a major bias for the study.

5. Results: As in the precedent point, the authors must justify the necessity and the significance of examining only 61.2 % of An. gambiae for Plasmodium infection, although they have been randomly selected.

6. Results: As in the precedent point, the authors must justify the necessity and significance of examining only 115 field collected mosquitoes for the kdr mutation.

7. Discussion: The authors stated that an effective measure to control malaria spread in Bankeng would be to distribute LLINs with a combination of a pyrethroid and the synergist piperonyl butoxide. Although the usage of this synergist in this experiment revealed a increase of the mosquito mortality, the authors did not mention the significant mortality percentage cut off known for the prescription of this combination. References would be of great help to sustain this recommendation, otherwise the authors should present it just as a justified suggestion, and not as a formal recommendation.

Minor revisions:

1. In the ‘Author’s details’ part of the manuscript, the authors wrote ‘Department of Medica Entomology…’, it should surely be ‘Department of Medical Entomology…’.

2. In the ‘Background’ part of the summary, the authors wrote ‘Cameroonian authorities have recently established irrigated rice projects across the country’, I suggest to replace ‘irrigated rice projects’ by ‘irrigated rice farming projects’. It sounds more clear and simple to understand.

3. In the ‘conclusion’ part of the summary, the authors wrote ‘… the implementation of the rice fields ; the present study…’, it should be ‘… the implementation of the rice field, the present study…’ with a ‘,’ instead of ‘;’

4. In Methods, part ‘mosquito collection’, the authors wrote ‘For each 2 hour of collection’. Shouldn’t it be ‘2 hours of collection’? If so, then please correct.

5. In Methods, part ‘PCR-species identification’, the authors wrote ‘using 0.34 mM of each primer and 1ul of genomic DNA’. Shouldn’t it be ‘using 0.34 mM of each primer and 1 µl of genomic DNA’? If so, then please correct.

6. In Methods, Insecticide susceptibility assays, authors wrote ‘2-5 days old F₀ female
mosquito'. Shouldn't it be ‘2-5 days old F₀ female mosquitoes’? If so, then please correct.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Medical entomology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 22 September 2020

https://doi.org/10.21956/wellcomeopenres.17621.r40394

© 2020 Djenontin A. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

**Aarmel Djenontin**

Université d'Abomey-Calavi/Centre de Recherche Entomologique de Cotonou (CREC), Cotonou, Benin

Elanga-Ndille and colleagues, in the manuscript “Entomological indicators of malaria transmission and insecticide resistance profile of *Anopheles gambiae* at the early phase of irrigated rice farming...
in the forest area of central Cameroon", attempt to catch up the absence of baseline data on malaria transmission before irrigated rice projects implementation across the Cameroon. Having seen that mosquito’s reproduction time is very short (about 2 weeks), data collected in the present study 6 months after the start of the rice project cannot be considered as baseline data for interpretation of the impact of this project on malaria transmission. The authors have to reconsider this statement in the manuscript to be in concordance with the title.

1. Having seen that mosquito's abundance has high spatial and temporal variation, how can a mosquito's collection at four points in a small village (250 inhabitants) during one month represent entomological indicators of malaria transmission and insecticide resistance profile of *Anopheles gambiae* in the forest area of central Cameroon?

2. Provide a study area map showing location of rice farming.

3. Check typography through the manuscript.

**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and is the work technically sound?**
Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**
Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**
Not applicable

**Are all the source data underlying the results available to ensure full reproducibility?**
No source data required

**Are the conclusions drawn adequately supported by the results?**
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Medical Entomology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.