Involvement of Anopheles nili in Plasmodium falciparum transmission in North Benin

Razaki A. Ossé1,2*, Filémon Tokponnon2,3, Germain Gil Padonou2,4, Aboubakar Sidick2, Rock Aïkpon2,5, Arsène Fassinou2,4, Come Z. Koukpo2,4, Wilfrid Sèwadé2, Bruno Akinro2, Arthur Sovi2,6, Melchior Aïssi7 and Martin C. Akogbéto2

Abstract

Background: Several studies carried out in Benin have shown the involvement of Anopheles gambiae sensu lato (s.l.), the Anopheles funestus group in malaria transmission, but none of them reported the contribution of the Anopheles nili group to the transmission of this disease. The current study investigated the question through an entomological cross-sectional survey performed in Northern Benin.

Methods: Mosquito samplings were performed in September and October 2017 in 4 villages located in two districts: Bambaba and Wodara (Kérou district) and, Péhunco 2 and Béké (Péhunco district). The collections were carried out indoors and outdoors using human landing catches (HLC) to assess the human biting rate (HBR) and pyrethrum spray catches (PSC) to evaluate the blood feeding rate and the blood meal origin using the ELISA test. All collected mosquitoes were morphologically identified and, the polymerase chain reaction (PCR) technique was used for molecular identification of sibling species of An. gambiae s.l., An. funestus group and An. nili group sporozoite index (SI) was also assessed by the ELISA test.

Results: Overall, An. gambiae s.l., An. funestus group and An. nili group were the three vectors found in the study area. A significantly higher human biting rate (HBR) was recorded in An. nili group (5 bites/human/night) compared to An. funestus group (0.656 bites/human/night) in the Kérou district (p < 0.0001). Anopheles gambiae s.l. displayed the highest HBR (26.19 bites/human/night) in the same district. The entomological inoculation rate (EIR) was 1.875 infected bites/human/month in An. nili group against 13.05 infected bites/human/month in An. gambiae s.l. and 0.938 infected bites/human/month in An. funestus group in Kérou. In Péhunco, the EIR was 1.02 infected bites/human/month in An. gambiae s.l. PCR results showed that An. nili sensu stricto (s.s.) and An. funestus s.s. were the only species of the An. nili and An. funestus groups, respectively. The anthropophagic character of An. gambiae s.l. was also highlighted.

Conclusion: This study provides useful information on the contribution of An. nili group as secondary vector to malaria transmission in northern Benin. Broader studies must also be carried out in a larger study area to assess the involvement of other Anopheles species to malaria transmission. This will aid to better plan malaria vector control interventions.

Keywords: Malaria, EIR, Anopheles nili, Contribution, Benin

*Correspondence: ossraz@yahoo.fr
1 Centre de Recherche Entomologique de Cotonou (CREC),
06 BP 2604 Cotonou, Benin
Full list of author information is available at the end of the article
Background
A wide range of human and animal diseases are transmitted by mosquitoes and those diseases remain a major source of death worldwide [1]. Malaria remains one of the most serious vector-borne diseases, affecting half of the world’s 7.4 billion people [2]. The proliferation of mosquitoes is promoted not only by ecological changes due to human activities (deforestation, public works, construction of dams, rice paddies, irrigation), but also by environmental parameters (rainfall, temperature and relative humidity), which also play a fundamental role in the level of transmission and the epidemiology of diseases [3, 4]. In intertropical Africa, malaria transmission is very heterogeneous due to eco-climatic variations [5]. Currently, five species of the parasite of the Plasmodium genus have been identified as responsible for malaria infection in humans [6]. Among them Plasmodium falciparum remains the most virulent species causing the deadly forms of malaria [7]. The Plasmodium species responsible for human malaria are mainly transmitted by primary vector species, such as Anopheles gambiae sensu lato (s.l.), Anopheles funestus group and Anopheles nili group [8, 9]. The existence of those different species complexes in an area represents a great challenge for malaria control programmes. Anopheles nili group has a wide geographical distribution in most of tropical Africa [10] and its preferential habitats are fast-flowing, upright-flowing streams, large rivers or dense shade along streams [7]. It is a group that includes four species, including An. nili sensu stricto (s.s.), Anopheles carnevalei, Anopheles somalicus and Anopheles ovengensis [8, 11].

In Benin, over the past two decades, the roles of An. gambiae s.l. and An. funestus group in the transmission of P. falciparum in several regions of the country have been studied by several authors [12–19]. Few of these studies have shown the presence of An. nili group in the different surveyed areas. Moreover, none of them has demonstrated its involvement in malaria transmission in Benin. It is in this context that a large population of An. nili group was collected during this cross-sectional survey conducted in the districts of Kérou and Péhunco located in the Atacora region. Thus, the possible involvement of this Anopheles species in the transmission of P. falciparum was investigated in the study area.

This study aims at providing information on vector species composition involved in malaria transmission in the areas of Kérou and Péhunco (northwestern Benin) where there is a lack of data.

Methods
Study area
This study was conducted from September to October 2017 in the districts of Kérou and Péhunco, both located in the north-western Benin (Fig. 1). The two districts have a Sudano-Guinean climate characterized by a rainy season, from mid-April to mid-October, and a dry season from mid-October to mid-April. They belong to an agro-ecological zone characterized by an average annual rainfall of 1000 mm. The average temperature varies between 25 in August and 31 °C in April.

The district of Kérou is irrigated by two large permanent rivers, the Mekrou and the Pendjari as well as of many tributaries from the Alibori river. Péhunco is drained by the river Mekrou and also by many rivers. The most important economic activity of these two districts is agriculture, especially with the cultivation of cotton, sorghum and cashew nuts [20].

Mosquito sampling and processing on the field
In each district, two villages were selected for mosquito collections, Wodara and Bambaba (respectively central and peripheral village of the Kérou district) and, Péhunco 2 and Béké (respectively central and peripheral village of the Péhunco district). Collections of mosquitoes were performed using both human landing catches (HLC) and pyrethrum spray catches (PSC).

HLCs were carried out in two houses per village. Four sessions of night collections were organized each month with one human sitting inside and another one outside of each house, bringing to a total of 16 human-nights/village/month and a total of 32 human-nights/district/month.

For PSCs, 10 bedrooms were surveyed in each district (5 in the central village and the remaining 5 in the peripheral village) to collect early in the mornings, all mosquitoes that have entered the houses the night before. Thus, aerosol bombs (Rambo®) containing 0.25% transfluthrin and 0.20% permethrin were sprayed in the houses and white canvas were spread on the floor for the collection of fallen mosquitoes.

Mosquitoes caught by both methods were morphologically identified using a mosquito identification key [21] and, the ovaries of the Anopheles vectors were dissected to determine the parity rate [22]. Anopheles vectors collected by PSCs were classified according to the physiological state of their abdomens (unfed, fed, half gravid, gravid) to determine the blood feeding rate. The Anopheles vectors collected with the two sampling methods were then stored in tagged eppendorf tubes containing silica gel and cotton for subsequent laboratory analysis.

Laboratory analysis
539 head-thoraxes of Anopheles vectors collected indoor and outdoor through HLC were crushed and then analysed by the ELISA tests for the determination of the circumsporozoite protein (CSP) positivity of Plasmodium
Fig. 1 Map of the study area
falciparum using the protocol of Wirtz et al. [23]. This allows the determination of the sporozoite index (SI). The legs, wings, and abdomens of these mosquitoes were used for DNA extraction to perform molecular species identification.

The PCR technique based on the protocols of Santomalazza et al. [24], Koekemoer et al. [25] and Kengne et al. [26] was used to identify the sibling species of An. gambiae complex, An. funestus group and An. nili group, respectively. In An. gambiae s.l., the presence of L1014F kdr and G119S ace-1 mutations was determined following the methods of Martinez-Torres et al. [27] and Weill et al. [28], respectively. Moreover, the blood meal origin was sought in the blood fed Anopheles mosquitoes collected by PSC, using a direct enzyme immunoassay (ELISA) according to the method of Beier et al. [29] with human, cattle, sheep, chicken and pig antibodies.

Data analysis

The results were processed and analysed using the R Core Team software (Version 3.5.1-2018) and Excel spreadsheets. The human biting rates (HBR=number of collected vectors/number of humans/number of nights), infection rates (Number of infected mosquitoes/total tested), entomological inoculation rates (EIRnight=HBR×infection rate; EIR_{month}=EIR_{night}×30) and parity rates (Number of parous mosquitoes/total tested) were calculated and compared between species of the same district. The Poisson test allowed us to compare the HBRs and EIRs between species in the district of Kérou between September and October 2017.

| Table 1 Mosquito species composition in Péhunco and Kérou between September and October 2017 |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Species                         | Péhunco Indoor  | Péhunco Outdoor | Kérou Indoor    | Kérou Outdoor   | Total           |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Anopheles gambiae s.l.          | 74              | 54              | 430             | 408             | 966             |
| Anopheles funestus group        | 0               | 1               | 9               | 12              | 22              |
| Anopheles nili group            | 0               | 0               | 51              | 109             | 160             |
| Anopheles pharoensis            | 0               | 0               | 0               | 1               | 1               |
| Anopheles ziemanni              | 0               | 0               | 14              | 33              | 47              |
| Anopheles brohieri              | 0               | 0               | 19              | 82              | 101             |
| Anopheles funestus s.l.         | 74              | 55              | 523             | 645             | 1297            |
| Aedes aegypti                   | 0               | 0               | 1               | 0               | 1               |
| Aedes vitatus                   | 0               | 0               | 2               | 2               | 2               |
| Culex quinquefasciatus          | 127             | 91              | 18              | 16              | 252             |
| Culex nebulosus                 | 0               | 2               | 0               | 0               | 2               |
| Culex tigripes                  | 1               | 0               | 0               | 0               | 1               |
| Mansonia africana               | 1               | 1               | 0               | 1               | 3               |
| Culicidae (sub total 2)         | 129             | 94              | 19              | 19              | 261             |
| Grand total                     | 203             | 149             | 542             | 664             | 1558            |

PCR results showed the presence of An. gambiae and An. coluzzii as sibling species of the An. gambiae complex in both Péhunco and Kérou. Anopheles gambiae was found in majority (96.43% and 78.43% at Pehunco and Kérou, respectively). In addition, all mosquito specimens of the An. funestus group (Péhunco and Kérou) and the An. nili group (Kérou) were found to be An. funestus s.s. and An. nili s.s., respectively (Fig. 2).

Biting behaviour, HBR, SI and EIR

In Kérou, the An. nili group was exophagic as the proportion of this species caught outdoors (68.13%: 109/160) was significantly higher than that recorded indoors (51.31%: 430/838) and the outdoor (48.69%: 408/838) biting behaviour of An. gambiae s.l. was similar (p=0.282), as was also observed for the An. funestus group [indoors proportion: 42.86% (9/21); outdoors proportion: 57.14% (12/21); p = 0.35]. However, in Pehunco, An. gambiae s.l. was endophagic [indoors proportion: 57.81% (74/128); Outdoors proportion: 42.19% (54/128); p = 0.011] (Table 1).

In the district of Kérou, the biting rate of An. gambiae s.l. (26 bites/human/night) was significantly higher than that of the An. nili group (5 bites/human/night) (p<0.0001). The biting rate of An. funestus group (0.656 bites/human/night) was significantly lower than that of An. gambiae s.l. and An. nili group (p<0.0001). The trend was the same between An. funestus group and An. gambiae s.l. in Péhunco (p<0.0001) (Table 2).
For the SI, no significant difference was recorded between the three vectors in the Kérou district ($p = 0.496$). In Péhunco, only An. gambiae s.l. was found positive for P. falciparum antigen (Table 2).

In Kérou, the indoors EIR of An. gambiae s.l. (20.156 infected bites/human/month) and An. funestus group (1.873 infected bites/human/month) were significantly higher than that recorded outdoors [An. gambiae...
s.l.: 6.12 infected bites/human/month (p < 0.0001); *An. funestus* group: 0 infected bites/human/month (p = 0.0005). In the same district, the indoor (1.913 infected bites/human/month) and the outdoor (1.839 infected bites/human/month) EIR of *An. nili* group was similar (p = 1). Furthermore, in the district of Kérou, the malaria transmission risk by *An. gambiae* s.l. (13.05 infected bites/human/month) was significantly higher than the malaria transmission risk of the *An. nili* group (1.875 infectious bites/human/month) (p = 0.0009) and the *An. funestus* group (0.938 infected bites/human/month) (p < 0.0001). It should be noted that the EIR of *An. nili* group was significantly higher than that of *An. funestus* group in Kérou (p < 0.0001) during the study period. In Péhunco, the indoor EIR for *An. gambiae* s.l. (1.943 infected bites/human/month) was significantly higher than that noted outdoor (0 infected bites per month) (p < 0.0001) (Table 2).

**Parity rate of *An. gambiae* s.l., *An. funestus* group and *An. nili* group**

Figure 3 shows the different parity rates with 88% [78.99–97.01], 57.14% [35.97–78.31] and 46% [32.19–59.81] for *An. gambiae* s.l., *An. funestus* group and *An. nili* group in the Kérou district, respectively. The parity rate in *An. gambiae* s.l. was significantly higher than that of *An. funestus* group (p = 0.004) and *An. nili* group (p < 0.0001). In Péhunco, the parity rate recorded for *An. gambiae* s.l. was 72% [59.55–84.45].

**Blood feeding rate and blood meal source in *An. gambiae* s.l.**

The blood feeding rate of *An. gambiae* s.l. collected inside houses after PSC was 63.16% in Péhunco against 60.53% in Kérou (p = 0.847). In both districts, the majority of *An. gambiae* s.l. vectors took their blood meal on human (91.30% in Kérou against 100% in Péhunco). In Kérou, 4.35% (1/23) of the collected *An. gambiae* s.l. blood fed on beef and, the same proportion on sheep (Table 3).

![Fig. 3 Parity rate in *An. gambiae* s.l., *An. funestus* s.s. and *An. nili* s.s. collected in Kérou and Péhunco](image)

**Table 3 Blood meal origin in *Anopheles gambiae* s.l. collected indoors in Kérou and Péhunco**

| Districts | T. collected | T. Blood fed | Blood feeding rate (%) | Blood meal origin in *An. gambiae* s.l. |
|-----------|--------------|--------------|------------------------|--------------------------------------|
|           |              |              |                        | N. Human (%) | N. Beef (%) | N. Sheep (%) | N. Pig (%) |
| Péhunco   | 19           | 12           | 63.16                  | 12 (100)    |             |             | 0          |
| Kérou     | 38           | 23           | 60.53                  | 21 (91.30)  | 1 (4.35)    | 1 (4.35)    | 0          |
| Total     | 57           | 35           | 61.40                  | 22 (62.86)  | 1 (2.86)    | 1 (2.86)    | 0          |

T, total of mosquitoes; N, number of mosquitoes having taken their blood meal on
L1014F kdr and G119S ace-1 allele frequencies in An. gambiae and An. coluzzii

The L1014F kdr mutation was found at very high frequencies in both Péhunco (86% and 100% respectively in An. gambiae and An. coluzzii) and Kérou (83% and 73% respectively in An. gambiae and An. coluzzii) (Table 4). In each district, the allelic frequencies of the L1014F kdr mutation were similar in An. gambiae and An. coluzzii (p > 0.05).

The G119S ace-1 mutation was also recorded in the two districts but at low levels with allelic frequencies of 5% and 0%, respectively, in An. gambiae and An. coluzzii in Péhunco and, 7.3% and 5%, respectively, in An. gambiae and An. coluzzii in Kérou (Table 4). No significant difference was also recorded between the allelic frequencies of the G119S ace-1 mutation of An. coluzzii and An. gambiae in both districts (p > 0.05).

Discussion

The study of malaria vectors in an area is a prerequisite not only to understand the epidemiology of the disease but also to implement a strategy for effective and targeted control of those vectors. In the current study, 12 mosquito species including 6 anopheline were identified in the two surveyed districts. Among these 6 Anopheles species, two (An. gambiae s.l. and An. funestus group) have already been implicated in malaria transmission in Benin [13, 17, 18]. During this cross-sectional study, they have already been implicated in malaria transmission in Benin [13, 17, 18]. These results corroborate those of Djouaka et al. [19, 34] who found An. funestus s.s. as the only species of the An. funestus group in the areas of Pahou and Kpomè in southern Benin. The results are also similar to works by Carnevale et al. [35], Dia et al. [36] and Adja et al. [31], who found An. nili s.s. as the only species in forest zone respectively in Cameroon, Senegal and Côte d’Ivoire.

The number of An. funestus group collected in both districts was low during the study period as was also reported by some authors in the area and around [32, 37, 38]. This could be justified by the scarcity of breeding sites favorable to the development of this species. Moreover, contrary to An. gambiae s.l., An. nili group was found to be exophagic (significantly higher biting activity outdoors compared to indoors) in Kérou. This low biting behaviour of An. nili group inside the dwellings had also been reported in Nigeria [39]. Kérou being a savanna area, this behaviour of An. nili group confirms the results of other authors who have shown that savanna populations of An. nili group are more exophagic and exophilic [40, 41].

In Kérou, each inhabitant receives 5 bites of An. nili group per night. This biting rate was significantly higher than the one of An. funestus group and not negligible for just four nights of collection. This reflects the presence of breeding sites favorable to the development of this species in the area. In fact, Kérou is close to several rivers, and the water level of these rivers is kept high for a good part of the year by the rain. This flow of water contributes to the development of several permanent mosquito breeding sites. This could probably explain the high density of the populations of the An. nili group in Kérou. As a result, in an area with multiple vectors, variation in ecological, spatial and temporal parameters directly influences the relative role of each species in malaria transmission [42].

Table 4 Allelic frequencies of L1014F kdr and G119S ace-1 mutations in Anopheles gambiae and Anopheles coluzzii in the Péhunco and Kérou districts

| Species       | N tested | L1014F Kdr | p value | G119S Ace-1 | p-value |
|---------------|----------|------------|---------|-------------|---------|
|               |          | RR RS SS F(L1014F Kdr) |         | RR RS SS F(G119S Ace-1) |         |
| Péhunco       | An. gambiae | 54 | 42 9 3 0.86 0.957 | 0 5 49 0.05 | 1 |
|               | An. coluzzii | 2 | 2 0 0 1 | 0 0 2 0 |         |
| Kérou         | An. gambiae | 41 | 30 8 3 0.83 0.439 | 0 6 35 0.073 | 1 |
|               | An. coluzzii | 11 | 7 2 2 0.73 | 0 1 10 0.05 |         |
species and allows this vector to maintain the transmission of malaria in both districts.

Another important aspect of the current study is the involvement of *An. nili* group in malaria transmission in Benin. Indeed, the SI of *An. nili* group was 1.25% in Kérou. This index is similar to that recorded by Carnevale et al. [35] in Cameroon and lower than the one of Elissa et al. [43] in Gabon and Adja et al. [31] in Côte d’Ivoire. The sporozoite indexes of *An. gambiae* s.l. in Kérou (1.7%) and Péhunco (0.9%) were lower than those reported by previous studies in different areas of Benin [16, 33, 37]. This could be due to the low number of tested mosquitoes in the short collection period covered by the current study as compared to previous records which considered a longer time period.

This study also showed malaria transmission by different mosquito species in the Kérou district. *An. gambiae* s.l. and *An. nili* group share in the indoors and outdoors malaria transmission. In *An. funestus* group where EIR has been observed indoors, no definitive conclusion can be drawn as to its participation in outdoor transmission since the collection period in this study is short. As previously found in several sites in Benin [13–18], *An. gambiae* s.l. also remains the primary vector of malaria transmission in Kérou (13.05 infected bites/human/month), followed by *An. nili* group (1.87 infected bites/human/month) and *An. funestus* group (0.94 bites/human/month).

The EIR by *An. gambiae* s.l. in Kérou was higher than those reported in a longitudinal study by Salako et al. [37] in similar biocological areas (Kandi and Gogounou) in the department of Alibori, Benin. In Péhunco, each inhabitant receives 1.02 infected bites of *An. gambiae* s.l. per month. The high transmission by *An. gambiae* s.l. in Kérou compared to both *An. funestus* group and *An. nili* group may be due to its significantly higher physiological age compared to the one of the other mosquito species (p < 0.05). The small number of *An. funestus* group collected in each district could explain its low level of malaria transmission. Findings of this study shows that no specimen of *An. nili* group and *An. funestus* group was collected indoors after PSC. This could be due to the low sampling of mosquitoes performed during our survey. *An. gambiae* s.l. which was the most frequent vector in the two districts takes mostly its blood meal on humans, thus confirming its anthropophagic nature [33].

Regarding the presence of resistance mechanisms in *An. gambiae* s.l., the L1014F *Kdr* mutation was found at very high frequencies in its two detected sibling species (*An. gambiae* and *An. coluzzii*) in the two districts. Contrary to the works of Gnanguenon et al. [44], Yahouédo et al. [45] and Akogbeto et al. [33] in some locations of southern and northern Benin, no significant difference was noted between the allelic frequencies of the L1014F *Kdr* mutation of *An. gambiae* and *An. coluzzii* in Kérou and Péhunco. This result corroborates those of Salako et al. [46] who also found no significant difference between the L1014F *Kdr* frequencies of *An. gambiae* and *An. coluzzii* in Djougou, Ségbana and Copargo, northern Benin. The trend was the same for the relatively low allelic frequencies of the G119S *ace-1* mutation of *An. gambiae* and *An. coluzzii* of the two study districts. However, the highest allelic frequencies of the G119S *Ace-1* mutation was recorded in *An. gambiae* in each zone with values ranging from 5 to 7.3%. In *An. coluzzii*, this frequency varied from 0 to Péhunco to 5% in Kérou.

**Conclusion**

In the present study, *An. gambiae* s.l., *An. funestus* group and *An. nili* group were the three most important vectors that transmit malaria in the Kérou district. This is the first report on the contribution of the *An. nili* group as a secondary vector of malaria transmission in Benin. This suggests broader studies involving other districts to determine if other *Anopheles* species also contribute to malaria transmission. This will help a better orientation of vector control interventions.

**Abbreviations**

HLC: human landing catches; HBR: human biting rate; PSC: pyrethrum spray catches; ELISA: enzyme-linked immunosorbent assay; PCR: polymerase chain reaction; SI: sporozoite index; EIR: entomological inoculation rate; CSP: circumsporozoite protein.

**Authors’ contributions**

RAO, FT, GGP, RA and MCA designed the study. RAO, AF and CK performed the fields activities. RAO, AS and WS carried out the laboratory’s activities. RAO, AF and CK performed the fields activities. RAO, AS and WS carried out the laboratory’s activities. RAO, FT, GGP, RA and RA critically revised the manuscript for intellectual content. All authors read and approved the final manuscript.

**Author details**

1. Ecole de Gestion et d’Exploitation des Systèmes d’Elevage, Université Nationale d’Agriculture, Kétou, Benin.  2. Centre de Recherche Entomologique de Cotonou (CREC), 06 BP 2604 Cotonou, Benin.  3. National Malaria Control Programme, Ministry of Health, Cotonou, Benin.  4. Faculté des Sciences et Techniques, Université d’Abomey-Calavi, Abomey-Calavi, Benin.  5. Ecole Normale Supérieure de Natitingou, Université Nationale des Sciences, Technologies, Ingénierie et Mathématiques, Natitingou, Benin.  6. Faculté d’Agronomie, Université de Parakou, Parakou, Benin.  7. Conseil National de Lutte contre le SIDA, la Tuberculose, le Paludisme, les Hépatites et les épidémies, Cotonou, Benin.

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**Competing interests**

The authors declare that they have no competing interests.
Availability of data and materials
The data supporting the conclusions of this article are included within the article. The raw data used in this study are available from the corresponding author upon reasonable request.

Consent for publication
Not applicable.

Ethics approval and consent to participate
This study was received the ethical approval by the Institutional Ethics Committee of CREC. The mosquito collectors gave their consent before participate to this study. They were checked up by treatment malaria after mosquito collection and were vaccinated against yellow fever.

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