Scientific Research Publishing

Advances in Entomology, 2022, 10, 99-109
https://www.scirp.org/journal/ae
ISSN Online: 2331-2017
ISSN Print: 2331-1991

Anopheles leesoni Evans 1931, a Member of the *Anopheles funestus* Group, Is a Potential Malaria Vector in Cameroon

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How to cite this paper: Kopya, E., Ndo, C., Djamouko-Djonkam, L., Nkahe, L., Awono-Ambene, P., Njiokou, F., Wondji, C.S. and Antonio-Nkondjio, C. (2022) *Anopheles leesoni* Evans 1931, a Member of the *Anopheles funestus* Group, Is a Potential Malaria Vector in Cameroon. *Advances in Entomology*, **10**, 99-109.
https://doi.org/10.4236/ae.2022.101008

Received: October 21, 2021
Accepted: January 10, 2022
Published: January 13, 2022

Abstract

Background: Understanding the biology of *Anopheles* malaria vector species is essential to planning effective and sustainable malaria control strategies in endemic countries. This study reported the implication of *Anopheles leesoni* in malaria transmission in Cameroon, Central Africa. **Methods:** Mosquitoes were collected in three localities from May 2015 to March 2018 using electric aspirators and Centers for Disease Control light traps (CDC-LT). *Anopheles funestus sensu lato* (*s.l.*) mosquitoes were identified as species using polymerase chain reaction assay (PCR). Furthermore, *Plasmodium falciparum* infection status was determined using the enzyme-linked immunosorbent assay (ELISA) method. **Results:** A total of 12,744 *Anopheles* mosquitoes were collected by electric aspirator (N = 4844) and CDC-LT (N = 7900). *Anopheles funestus s.l.* (86.95%) was the major species and the main malaria vector in rural savannah and rural forest sites followed by *A. gambiae s.l.* (13.05%) whereas in urban areas, *A. gambiae s.l.* was by far the most abundant representing 91.45% of *Anopheles* mosquitoes collected. Two members of the *A. funestus* group were identified among 1389 analysed by PCR: 1307 *A. funestus sensu stricto* (*s.s.*) (94.10%) and 82 *A. leesoni* (5.9%). *Plasmodium falciparum* infection rate was 21.04% in *A. funestus s.s.* For the first time, *A.*

leesoni was found positive for *P. falciparum* (infection rate: 10.98%) in Cameroon. **Conclusion:** A very high *P. falciparum* infection rate was observed in this study in *A. funestus s.s.*, highlighting its high implication in malaria transmission in Cameroon. Furthermore, the detection of *P. falciparum* infection in *A. leesoni* calls for more attention towards this neglected vector species.

Keywords

Anopheles funestus Group, *Anopheles leesoni*, *Plasmodium falciparum*, Malaria, Transmission, Cameroon

1. Introduction

In Africa, the most important and widespread vectors of malaria belong to the *An. gambiae* complex and the *A. funestus* group [1] [2] [3]. Adult members of these two complexes/groups are difficult to distinguish morphologically [4] [5], necessitating molecular techniques for accurate identification [1] [6] [7].

Within the *A. funestus* group, *A. funestus s.s.* is the only member that plays a significant role in the transmission of human malaria throughout the African continent, but other species of *A. funestus* group have been naturally found infected with *P. falciparum* [8]. This mosquito is widely distributed throughout tropical Africa and its breeding site is permanent or semi-permanent. Its activity extends even during the dry season where other malaria mosquito vectors, such as *A. gambiae s.l.* are usually less abundant [9]. As for other members of the *A. funestus* group, some reports indicated that *A. rivulorum* may be involved in malaria transmission in some situations [8] [10] and *P. falciparum* has been reported from *A. parensis* and *A. leesoni* [11]. *A. vaneedeni* has been experimentally infected with *P. falciparum* [12] which has recently been isolated in natural populations of this species in South Africa [13]. No reports of any involvement in malaria transmission for the remaining members of the *A. funestus* group were found. Despite their morphological similarity, the species of *A. funestus* group shows different vectorial capacities and then different malaria transmission capacities. Therefore, there is a necessity to determine the predilection place of action of each species in order to readapt malaria vector control decisions and operations, focusing on really affected areas and making vital commitments in all African countries where financial resources relating to related malaria control are limited.

Historical evidence suggests that in order to conduct an efficient vector control program, there is a necessity to identify and distinguish vector species from non-vector species. Control measures against *A. funestus s.s.*, which is an anthropophilic and endophilic vector, favour exophilic members of the *A. funestus* group, increasing their density [14]. For example, in South Africa [12] [15], Kenya [16], and Tanzania [14] [17], indoor spraying used to eliminate *A. funestus s.s.*

was followed by an upsurge of "*funestus* look-alike" specimens, contributing to the failure of the control program. However, further investigation revealed that these mosquitoes belong to *A. vaneedeni*, *A. parensis*, *A. rivolurum*, or *A. leesoni*. Each of them is occasionally or rarely implicated in the transmission of malaria to humans, and their zoophilic and exophilic habits probably reduce exposure to insecticides.

Reliable species identification is indeed important to assess the relative role played by each species in the transmission of Plasmodium and improve our ability to evaluate the efficacy of vector control measures implemented in areas where several species of the *A. funestus* group are present. In the past, species identification has mainly been performed using either morphological or cytogenetic methods. However, the development of PCR-based methods has greatly facilitated the identification of species in the group [7] [18]. It is commonly asserted that malaria transmission in Africa is maintained by members of the *A. gambiae* complex [19]. However, in several parts of the continent, other mosquito species contribute to the transmission of the parasite, including *A. funestus s.l.* and *A. nili*. Previous studies in Cameroon defend that *A. funestus s.s.* is the main, if not the only vector of the *A. funestus* group responsible for transmission of malaria parasites [7] [20] [21] [22]. Although *A. leesoni* [7] [20] [21] have been found in several malaria foci, their role in the transmission of malaria in Cameroon has not been further studied.

In this paper, we provide evidence incriminating *A. leesoni* in the transmission of malaria in Cameroon. Demonstrating at the same time the presence of two species of the *A. funestus* group in this country, where malaria transmission is a serious public health problem.

2. Methods

2.1. Study Area

The study was carried out in three sites belonging to the forest and savannah domains of Cameroon (**Figure 1**).

Mebelong (6˚46'N, 11˚70'E) is located in the Adamawa region, approximately 350 km from Yaoundé, the capital city of Cameroon. The village is situated at the vicinity of a lake that represents a potential breeding site for *A. funestus s.s.* mosquitoes throughout the year [23]. The climate is Sudano-Guinean characterized by an eight-months rainy season from March to October, and a dry season of four months extending from November to February [24].

Obout (12˚53'N, 35˚7'E) and Yaoundé (3˚52'N, 11˚27'E) are located about 30 km apart within the forest regions area of the Centre region. The climate is alike to that of Equatorial Guinea, characterized by two rainy seasons extending from August to October, and from April to June. There are also two dry seasons running from November to April and from June to July [24]. The village Obout is situated in rural zone and is surrounded by an evergreen forest. Within the village, there are several fish ponds bordered with emergent vegetation suitable for the development of Anopheles mosquito larvae, particularly those of *A. funestus* group.

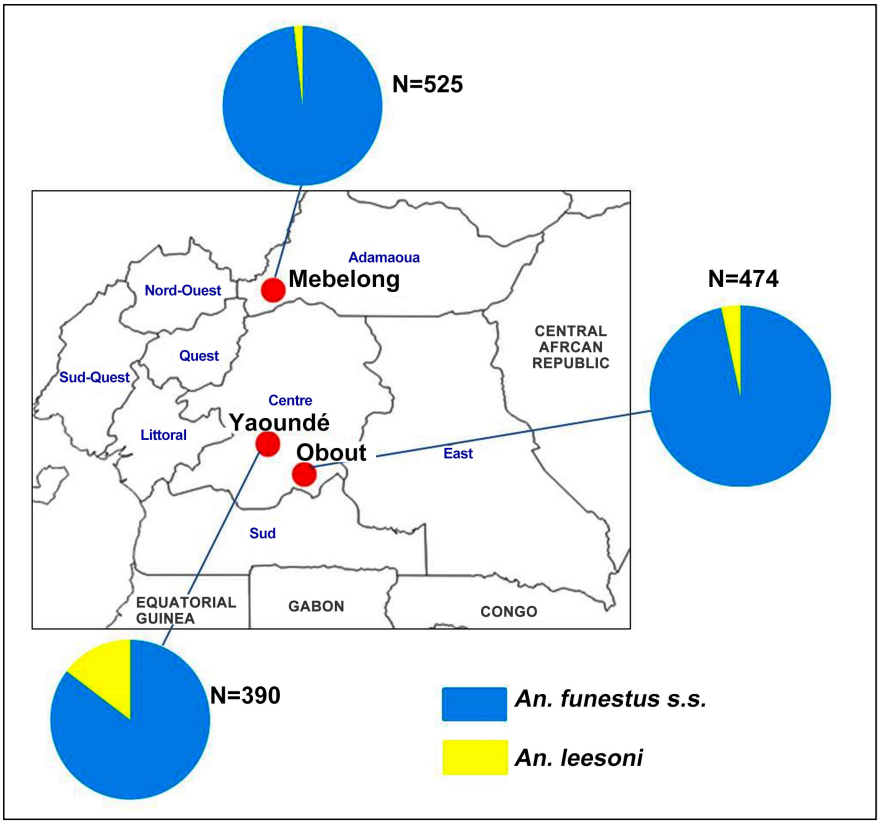

Figure 1. Species composition within the *A. funestus* group in the three localities (N: number of mosquitoes identified by PCR).

The town Yaoundé is made up of wetlands and a degraded forest surrounding the city. There are also lakes and fish ponds suitable for the development of anopheline mosquitoes. Yaoundé features an equatorial climate with two rainy seasons extending from March to June and from September to November lasting 7 to 8 months [24].

2.2. Ethical Consideration

The study was approved by the Cameroonian national ethical committee for research on human health (statement N˚ 2015/01/535/CE/CNRERSH/SP). Verbal informed consent was obtained from each head household before the team entered their houses for mosquito collection.

2.3. Mosquito Collections and Identification

Mosquitoes were collected from May 2015 to December 2017 in Obout and Mebelong and from May 2017 to March 2018 in Yaoundé.

In Obout and Mebelong, indoor resting mosquitoes were collected in human dwellings in the morning 10 to 15 houses, between 7:00 and 10:00 AM using electric aspirators (Rule In-Line Blowers, Model 240) whereas in Yaoundé mosquitoes were collected from 6:00 PM to 6:00 AM using CDC light Traps placed indoors and outdoors in 10 to 15 houses. After species identification using mor-

phological keys [4] [5], only mosquitoes belonging to *A. funestus* group were included in the subsequent analysis.

2.4. Molecular Identification of *Anopheles funestus* Members

Molecular identification of specimens of *A. funestus* group was performed following the species-specific protocols described by [21] and [7]. Abdomen, legs and wings were used for genomic DNA extraction as described previously [25]. The primers contained in **Table 1** were used. A final 25 µL reaction volume of PCR contained 2.5 µL of 10× buffer including 15 mM $MgCl_2$, 5 pmol of each primer, 200 µM of each dNTP, and 0.5 units of *Taq* polymerase unit. Amplification started with an initial denaturation step at 94˚C for two minutes, followed by 36 cycles of denaturation at 94˚C for 30 seconds, annealing at 45˚C for 30 seconds, and elongation at 72˚C for 40 seconds, with a final extension step at 72˚C for five minutes. The PCR products were loaded and visualized on regular 1.5% agarose gels stained with ethidium bromide.

2.5. Detection of *Plasmodium falciparum* Infection

Head and thorax of each female mosquito were subjected to indirect enzyme-linked immunosorbent assay (ELISA) for the presence of *P. falciparum* circumsporozoite protein (CSP) using monoclonal antibodies 2A10 as described by Wirtz *et al.* [26]. One positive control and ten negative controls were added to each microtitre plate. Negative controls were head and thorax of unfed *A. gambiae s.s.* (Kisumu) from laboratory colonies maintained at OCEAC. Absorbance was measured at 405 nm using a microtitre plate reader (BioTek ELx800, Swindon, UK). The cut-off value for positive specimens was estimated at twice the mean value of the negative controls.

2.6. Statistical Analysis

Infection rates were determined as the percentage of mosquito species samples found positive for *P. falciparum* over the total number of specimens tested.

Table 1. Sequences of primers used for molecular identification of *An. funestus* species.

Primers	Sequences	Species	Band size (bp)
UV	5'TGTGACTGCAGGACACAT3'	Universal	-
FUN	5'GCATCGTGAGGTTAATCATG3'	*An. funestus*	505
VAN	5'TGTCGACTTGGTAGCCGAAC3'	*An. vaneedeni*	587
RIV	5'CAAGCCGTTCGACCCTGATT3'	*An. rivulorum*	411
PAR	5'TGCGGTCCCAAGCTAGGTTC3'	*An. parensis*	252
LEE	5'TACACGGGCGCCTGATAGTT3'	*An. leesoni*	146
RIVLIKE	5'CCGCCTCCCGTGGAGTGGGGG3'	*An. rivulorum-like*	313

3. Results

3.1. *Anopheles* Mosquito Population

A total of 12,744 resting *Anopheles* mosquitoes were collected during the study period including 7857 *A. gambiae s.l.* and 4887 *A. funestus s.l.* (Table 2). *Anopheles funestus s.l.* was by far the most abundant in Obout and Mebelong representing 74.42% and 97.12% of the total *Anopheles* mosquitoes caught respectively. By contrast, *A. gambiae s.l.* was the most frequent species in the city of Yaoundé (91.46%).

3.2. *Anopheles leesoni* Abundance and Distribution

The *Anopheles funestus* group, as revealed by the molecular identification of 1389 individual mosquitoes, was composed of two species, including 1307 *A. funestus s.s.* (94.10%) and 82 *A. leesoni* (5.90%). Both species were found in all three localities (Figure 1). However, the proportion of *A. leesoni* was higher in Yaoundé (57/390: 14.62%) than in Obout (16/474: 3.38%) and in Mebelong (9/525: 1.71%).

3.3. *Plasmodium* Infection Rates

Plasmodium falciparum infection rates are given in Table 3. Of a total of 1389 head and thorax analyses, 284 were positive, corresponding to a high global circumsporozoite rate of 20.45%. Among the mosquitoes tested, 21.04% (275/1307) were infected with *A. funestus s.s.* and 10.98% (9/82) for *A. leesoni*. Although the infection rate of *A. funestus s.s.* appeared higher in Obout and Mebelong compared to *A. leesoni*, this difference was not statistically significant ($P > 0.05$). No difference in terms of infection was observed between both species in Yaoundé ($P = 0.11$).

Table 2. Number of *A. funestus s.l.* and *A. gambiae s.l.* mosquitoes collected in Obout, Mebelong and Yaoundé.

Species	Obout	Mebelong	Yaoundé	Total
A. funestus s.l.	1615 (74.42%)	2597 (97.12%)	675 (8.54%)	4887 (38.35%)
A. gambiae s.l.	555 (25.58%)	77 (2.88%)	7225 (91.46%)	7857 (61.65%)
Total	**2170 (100%)**	**2674 (100%)**	**7900 (100%)**	**12,744 (100%)**

Table 3. Circumsporozoite protein (CSP) rates of *A. funestus s.s.* and *A. leesoni* mosquitoes from the study locations.

Localities	*A. funestus s.s.*			*A. leesoni*		
	Tested	Positive	Infection rate ($CI_{95\%}$)	Tested	Positive	Infection rate ($CI_{95\%}$)
Obout	458	155	33.84% (28.72 - 39.61)	16	3	18.00% (3.87 - 54.8)
Mebelong	516	107	20.74% (16.99 - 25.06)	9	1	11.11% (0.28 - 61.91)
Yaoundé	333	13	3.90% (2.8 - 6.68)	57	5	8.77% (2.84 - 20.47)
Total	**1307**	**275**	**21.04% (18.63 - 23.68)**	**82**	**9**	**10.98% (5.02 - 20.84)**

4. Discussion

The control of Anopheles vector populations is the pillar of malaria elimination strategies. Identifying primary disease vectors and understanding their biology and geographic distribution is crucial to plan efficient control strategies. For several decades attention has been focused of mosquitoes from *A. gambiae* complex which have for a long time been considered as the most efficient malaria vector throughout Africa continent. However, the recent increase of interest in other Anopheles species, such as those from *Anopheles funestus* group led to the change of this paradigm. Similar to this study *A. funestus s.s.* was repeatedly reported to be widespread and highly infected with *P. falciparum*, thus playing a major role in malaria transmission in East, Central and West Africa.

If knowledge and control of major vector species using insecticide and insecticide-treated tools successfully contributed to malaria reduction over the past decade [27], the real challenge for malaria elimination and eradication could arise from secondary vectors that sustain residual malaria transmission in the absence of primary vectors. Unfortunately, little is known regarding the distribution and biology of such secondary vectors.

In this study, we have demonstrated that *A. leesoni* is sympatric with *A. funestus s.s.* in forest and humid savannah ecosystems in Cameroon and was infected with *P. falciparum* in all the study sites. Previous studies have already reported the presence of *A. leesoni* in Yaoundé but not in Obout and Mebelong. However, to our knowledge, this is the first time this species has been incriminated as a malaria vector in Cameroon [28]. In other regions of the continent, there are some reports [11] of the possible carriage of *P. falciparum* parasites by *A. leesoni*, but there is no or little evidence of its role as a secondary malaria vector. Other members of the *A. funestus* groups, such as *A. rivulorum* and *A. vaneedeni*, were also found infected by malaria parasites in laboratories and in nature. *Anopheles rivulorum* has been implicated in malaria transmission or found to harbour *P. falciparum* parasites in Kenya [10], Tanzania [8] [11] and Zambia [29]. *Anopheles vaneedeni* has been experimentally infected with *Plasmodium* in the laboratory [12] and was recently found infected in nature [13].

Although we didn't assess *A. leesoni*'s feeding behaviour by determining the origin of the blood meal in the abdomens of mosquitoes, the fact that blood-fed *A. leesoni* was found resting inside human dwellings suggests that this species is endophilic and anthropophilic. Previous research by Temu *et al.* [11] in Tanzania revealed a preference for humans (81.8%) over goats (0%), with the species also resting inside human dwellings in Kenya [29] and West Africa [30].

Relatively high rates of infections of *P. falciparum* were detected in *A. funestus s.s.* and *A. leesoni* collected in our study area. This is the first report on *Plasmodium* infection in *A. leesoni* in Cameroon. Although this vector has been reported from different sites in East Africa and has been shown to play a major role in malaria transmission in Africa, no information is so far available on its infection with malaria parasites in Central Africa (Cameroon). Such high infection rates in non-vectors should be interpreted with caution because none of the

previous studies have reported any samples of *A. leesoni* infected with the *Plasmodium* parasite, either by salivary gland dissections or ELISA detection methods [4] [21]. While we cannot exclude the possibility that the ELISA is detecting sporozoites in the salivary glands alone, it is likely that the method is also picking up parasites in the thoracic hemocele. Therefore, unlike salivary gland dissections, ELISA does not guarantee that the mosquito is infectious unless it is carried out on the salivary glands themselves. Further studies are required involving detection of parasites by ELISA or PCR performed on salivary glands dissected from wild members of the *A. funestus* group.

Although it is in low abundance in this study, *A. leesoni* appears to transmit malaria as well as *A. funestus s.s.* in Cameroon. As reported in Tanzania [11], more than one species within the *A. funestus* group was found infected with *P. falciparum*, therefore sustaining the need to identify and adjust the list of malaria vectors that belong to species groups or complexes in order to establish areas of sympatric existence and to assess the role played by each species in malaria transmission. This information will improve our ability to evaluate the efficient and strategic planning of vector control measures.

In conclusion, since mosquito abundance displays temporal and spatial fluctuation and since more than one species within the *A. funestus* group was found infected with *P. falciparum*, it is important to characterize the spatial distribution of the *A. funestus s.s.*, *A. rivulorum*, and *A. leesoni* according to the malaria endemicity rate. Among members of the *A. funestus* group, the species composition and species diversity are likely to differ locally, leading to significant impact on efficiency of malaria vector control management.

Acknowledgements

This work was supported by a Wellcome Trust Training Fellowship in Public Health and Tropical Medicine (WT 102543/Z/13/Z) to CN and a Wellcome Trust Fellowship Senior in Public Health and Tropical Medicine (WT 202687/Z/16/Z) to CAN.

Authors' contributions

CAN and CN conceived and designed the study protocol; EK, LDD and LN participated in data collection and laboratory analyses; CAN, CN, PAA, FN and CSW critically revised the manuscript; EK, CN and CAN interpreted, analysed data, and wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Abbreviations

CDC: Centre for Disease Control and Prevention; ELISA: Enzyme Linked Immunosorbent Assay; PCR: Polymerase Chain Reaction; *s.l.*: *sensu lato*; *s.s.*: *sensu stricto*; *A.*: *Anopheles*