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Malaria Transmission and Vector Resistance to Insecticides in a Changing Environment: Case of Simbock in Yaoundé-City, Cameroon

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Ecological upheavals resulting from uncontrolled urbanization can lead to significant changes in vector borne diseases' profiles, thus requiring a thorough revision of their prevention and control strategies. The current study aimed at characterizing malaria vector populations in the Simbock neighborhood of Yaoundé-city (Cameroon), in relation to its urbanization scheme. Adult mosquitoes were captured by human landing catches (HLC) in- and outdoors prior to (2000–2006) and during infrastructural development (2014–2016). Anophelines were morphologically identified and analyzed for *Plasmodium (P.) falciparum* circumsporozoite protein detection using the ELISA technique. Species of the *Anopheles (An.) gambiae* complex were identified using SINE-PCR. Adult *An. gambiae* s.l. from larvae collected between 2014 and 2017 were tested for susceptibility to insecticides (0.1% bendiocarb, 4% DDT, 0.75% permethrin and 0.05% deltamethrin) with or without piperonyl butoxide (PBO) synergist, using WHO standard bioassays. The Hot Oligonucleotide Ligation Assay was used to detect the knockdown resistance (kdr) L995F/S mutations. Overall, nine malaria vector species were identified in 2000-2006, mostly *An. moucheti* (49%), *An. nili* (13.5%) and *An. gambiae* s.l. (12%); the six remaining species were represented at less than 3% each. However, only three species were found in 2014-2016, with increasing proportions of *An. gambiae* s.l. (67%) and *An. funestus* (32%) (P<0.0001). *An. gambiae* s.l. consisted *An. coluzzii* (> 85%) and *An. gambiae* (<15%) species during the two study periods. *Plasmodium falciparum* infection rates were 2.1% and 1.0% in 2000-2006 and 2014-2016 respectively (P=0.4), with decreasing entomological inoculation rates (EIR) from 0.34 infective bites per man per night (ib/m/n) to 0.02 ib/m/n (P<0.0001). *Anopheles gambiae* s.l. was resistant to DDT and permethrin [<40% mortality rates (MR)], and deltamethrin (65-89% MR), but fully susceptible to

bendiocarb (100% MR). Pre-exposure of mosquitoes to PBO resulted in 90-100% MR to deltamethrin but not to permethrin. Furthermore, the two kdr L995F/S resistance alleles were recorded at 0.64 and 0.006 frequencies respectively. This study highlights a shift from rural to urban malaria transmission in Simbock, coupled with DDT and pyrethroid resistance in *An. gambiae* s.l. Combination vector control interventions, e.g., PBO nets and bendiocarb indoor residual spraying are needed in such areas.

Keywords: insecticide resistance, urbanization, malaria transmission, malaria vectors, Anopheline mosquitoes, environmental change

INTRODUCTION

African cities experience a rapid demographic expansion as a result of various factors among which rural to urban migration, improved life expectancy, high birth rates and developmental projects (1). In order to accommodate the fast-growing human populations, rural areas surrounding the cities have turned to urban neighborhoods. These new neighborhoods are characterized by uncontrolled habitat constructions and landscaping activities, leading to important environmental changes.

In general, environmental modifications occurring naturally or through human activities, disrupt the ecological balance thus creating new habitats for living organisms (2). These upheavals have deep consequences on vector biology whereby some competent species may emerge to the detriment of the others, thus influencing the transmission profile of vector-borne diseases (3). Previous studies have revealed deep changes in the epidemiology of vector-borne diseases (VBDs) such as dengue, onchocerciasis, and particularly malaria (4). Malaria is a major public health concern in Africa. From 2019 to 2020, the incidence of malaria cases increased from 213 million to 228 million, highlighting the need to intensify prevention and control efforts (5). Cameroon is among the eleven countries mostly affected in the world (5) with about eighteen anopheline species involved in the transmission of *Plasmodium* parasites. Major vector species are *An. gambiae*, *An. coluzzii*, *An. arabiensis*, *An. funestus*, *An. nili*, *An. moucheti* known for their high anthropophilic behaviour (6–9). Other species considered as secondary vectors include *An. carnevalei*, *An. coustani*, *An. hancocki*, *An. leesoni*, *An. marshallii*, *An. melas*, *An. paludis*, *An. pharoensis*, *An. ovengensis*, *An. wellcomei*, *An. rufipes*, and *An.* ziemanni (10–15). Some of these vectors exhibit the ability to adapt to changing environmental conditions (16–18), even though each of them shows ecological preferences. For example, *An. coluzzii* which has a strong preference for unpolluted waters (19) has been found breeding in highly polluted water sources after urbanization in several countries in Africa (20–22), moreover, Mbakop et al. (23) showed the emergence of *An. paludis*, responsible for the majority of malaria transmission in Nyabessan, Cameroun during the construction of the hydroelectric dam.

Malaria has generally been considered as a disease of rural areas, but many factors linked to a rapid and uncontrolled urbanization are increasing malaria transmission in cities across Africa (24, 25). The expansion of malaria transmission to urban areas is of particular concern to malaria control programs since populations in these areas are likely to be at higher risk of the development of severe malaria due to the lack of protective immunity and the spread of drug resistant *Plasmodium* strains.

In Yaoundé, malaria transmission is considered holo-endemic and seasonal, with *An. gambiae* as the main vector (22, 26). Average annual prevalence of *P. falciparum* in the general population is estimated to increase from 34% in the city centre to 50% in the periphery (27). Malaria prevention is essentially based on vector control. Chemical vector control in the Yaoundé city began in 1950s with DDT indoor spraying campaigns; then, in 1990 there was the distribution of impregnated mosquito nets with deltamethrin, in a few households (28). Later on, three other mass LLIN distribution campaigns were conducted in 2004, 2011 and 2015 (29, 30). In addition, to remedy the increase in culicidian nuisance induced by uncontrolled urbanization (31), the populations of Yaoundé also report the use of spirals, aerosol bombs, and mosquito repellent ointments (32). Following these interventions, bed net ownership in Yaoundé was estimated at 73.8% (33), with subsequent reduction of malaria incidence. However, since 2017, the country is witnessing the resurgence of malaria with a rise of cases and deaths up to 6612000 and 14448 respectively in 2021 (34), thus requiring a « High Burden to High Impact » approach. Within this approach, the arsenal for malaria prevention includes the promotion of ITN use, intermittent preventive treatment for pregnant women and seasonal malaria chemoprevention for children aged 3–59 months in the humid tropical regions.

Indeed, during the last two decades, Cameroon has been facing anarchic urbanization, with the extension of cities to their outskirts. This extension leads to a demographic explosion couple with insalubrity and increased nutritional needs. This situation results in permanent stagnant waste water and crop cultivation in urban lands (35, 36). The Simbock neighborhood in the city of Yaoundé is a perfect illustration of this phenomenon. Previous studies conducted between 1999 and 2009 has revealed deep changes in the epidemiology of malaria following the emergence of highly anthropophilic vectors such as *An. gambiae* s.l. and *An. funestus* (37–41). The most recent malaria entomological study in Simbock was from 2000 to 2001 (41), while the habitat and the ecology of the environment have deeply

changed over time. Moreover, the susceptibility of emerging vector species to insecticides was not assessed. As such, a better understanding of the malaria entomological profile in such area under modification could improve and contextualize the implementation of new vector control interventions.

The current study aimed at assessing the entomological parameters of malaria transmission and the susceptibility of malaria vectors to insecticides in Simbock, a former rural area turned to a neighborhood of the Yaoundé, the capital city of Cameroon.

MATERIALS AND METHODS

Study Design

This study included retrospective and prospective surveys of malaria transmission indicators and four annual cross-sectional surveys of An. gambiae s.l. susceptibility to insecticides from 2014 to 2017. Retrospective data (2000–2006) were retrieved from previous entomological surveys stored in a mosquito data base in the malaria research laboratory at Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale (LRP/OCEAC), while prospective data (2014–2016) were collected through surveys according to thesame data collection scheme of retrospective data.

Study Area

The study was carried out in Simbock (3° 50'N, 11° 30'E, 750 m) located about 10 km from the divisional head quarter of Yaoundé, the capital city of Cameroon (**Figure 1A**). The climate in this area is of equatorial type characterized by two dry seasons (December-February and July-August) and two rainy seasons (March-June and September-November). The average annual temperature is 23°C and the mean annual rainfall 1,727 mm (42). Simbock is watered by the Mefou river, exhibiting in its course swamps which constitute permanent breeding sites of mosquitoes. Furthermore, there are some artificial fish ponds.

Before the 2000s, this locality had a rural landscape with the rate of built-up land (RBL) < 40%. Houses were surrounded by the forest, built with mud, roofed with straw, without ceiling. The main track through the village was unpaved (**Figure 1B**). At that time, the mean entomological inoculation rate was 0.69 infected bites per man per night (ib/m/n), and the plasmodial species present were *P. falciparum* and *P. ovale* (41). In order to facilitate the traffic between Douala the economic capital and Yaoundé the political capital of the country, a road construction project *via* Simbock was launched in 2008. Following this project, this locality has been experiencing overcrowding from 300 inhabitants in 2002 (39) to around 10,000 inhabitants in 2021 (43) and anarchic housing multiplication with conditions commonly better than those of the retrospective period (**Figure 1C**). Most houses are built with concrete floors and walls, ceiling under the roof and screened windows. Socio-economic development has been accompanied by the poor water drainage, responsible for the creation of permanent breeding sites characteristic for the proliferation of mosquitoes of the genus *Culex*. Vegetable crops, often responsible for the creation of anopheline breeding sites, are not practiced there; but there are ponds that already existed and are in the process of eutrophisation because they are poorly maintained. Most anophelines breeding sites are temporary and are numerous during the rainy season. Most of the inhabitants work in the city center or in the shops built along the main axis.

Study Periods

This study included retrospective and prospective surveys of malaria transmission indicators and four annual cross-sectional surveys of *An. gambiae* s.l. susceptibility to insecticides from 2014 to 2017. Details on the periodicity of the adults and larval collections are summarized in **Table 1**. Retrospective data were drawn from the malaria entomological data base resulting from a study conducted by the "Laboratoire de Recherche sur le Paludisme" (LRP) of Organisation de Coordination pour la lutte Contre les Endémies en Afrique Centrale (OCEAC) between January 2000 and November 2006. Prospective data on malaria transmission were collected between 2014 and 2016.

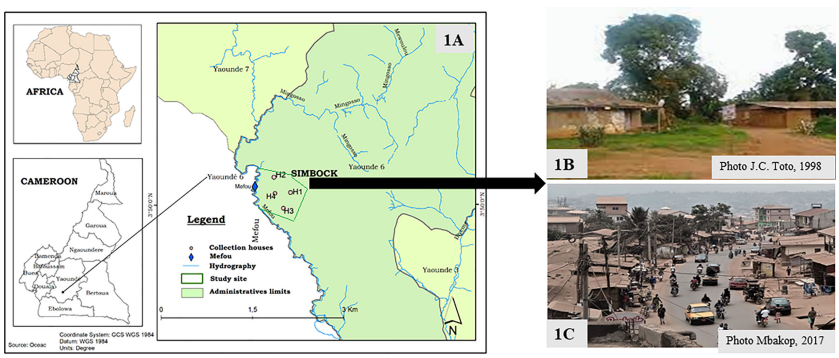

FIGURE 1 | Map of Yaoundé subdivisions showing Simbock and his configuration before the 2000s and following the urbanization process. **(A)**: Map of Yaoundé subdivisions showing Simbock, **(B)**: Before urbanization, **(C)**: Following the urbanization process, H1-H4: Mosquito collection houses.

TABLE 1 | Periods of entomological surveys.

Type of study	Retrospective				Prospective						
Activity	Adult mosquito collections				Adult mosquito collections			Larval collections			
Year	2000	2001	2004	2006	2014	2015	2016	2014	2015	2016	2017
Nb pnc	16.6	34.5	25.0	12	18	12	12	–	–	–	–
Month											
January	X	X							X		
February		X									
Mars	X	X									
April	X	X	X			X					
June										X	
July	X		X				X	X			
September	X										
November	X			X	X						
December	X										X
Total round of collections	7	4	2	1	1	1	1	1	1	1	1

Nb pnc, Number of person–night (volunteers) per month used for adult mosquito collections.

Mosquito Collection and Morphological Identification

Adult mosquitoes were collected *via* human landing catches (HLC) both during the retrospective (2000–2006) and the prospective period (2014-2016). Mosquito collections were performed as described in (14). In 2000-2006, mosquito collections were performed in two to four households (collection points) randomly selected, for two consecutive nights per survey period, including 14 surveys in total (7 surveys in 2000, 4 in 2001, 2 in 2004 and 1 in 2006) (**Table 1**). In 2014-2016, mosquitoes were collected in 3 households selected among those of the retrospective period, also for two consecutive nights per period of survey, including 3 surveys in all (1 survey per year). The surveys were cross-sectional whether during the retrospective or the prospective period, but covered the 2 dry seasons and the 2 rainy seasons. Mosquito collections were carried out per night by 12 to 34 volunteers during the retrospective period and 12 to 18 volunteers during the prospective period, working in two teams, the first team captured adult mosquitoes from 19:00 to 01:00 and the second team collected from 01:00 to 05:00. At each selected house, one volunteer captured indoors while another captured outdoors. To avoid bias, the volunteers rotated between indoors and outdoors every two hours. Mosquitoes collected were placed into separate bags, labelled according household, position and time of collection; a supervisor collected the bags at the end of each hour of catches.After collections, mosquitoes were morphologically identified using the standard identification keys (44, 45) and individually stored in labeled 1.5 mL Eppendorf tubes containing silica gel desiccant for further analysis.

For insecticide resistance assessment, anopheline larvae and pupae samples were collected from water bodies, including puddles disseminated throughout the quarter, gutters and pools of water strewing the Mefou river. Samples were pooled and transferred at the OCEAC Malaria Research insectary for rearing. Mosquitoes were reared in the laboratory at optimal temperature (28–30°C) and relative humidity (70–80%).

Laboratory Analyses and Testing

Each collected adult mosquito was dissected into two parts, the head-thorax region and carcasses.

Plasmodium falciparum Detection

Anopheline head and thorax were used for the detection of P. falciparum circumsporozoite protein (CSP) using the ELISA CSP technique (46, 47).

Molecular Identification of Species

Carcasses (legs/wings/abdomen) were used for DNA extraction as described by Collins et al. (48). PCR was then performed on the DNA template using An. gambiae s.l. species diagnostic primers in a total reaction mix of 25 µl, according to the protocol of Santolamazza et al. (49). The PCR product was visualized on 1.5% agarose gel to determine the sibling species.

Insecticide Susceptibility Tests

Female adult mosquito resulting from larval collections and aged 2-5 days were morphologically identified as *An. gambiae* s.l (44, 45). and used for susceptibility tests. Other susceptibility tests were carried out with female mosquitoes of the Kisumu susceptible reference strain of *An. gambiae*, reared in the OCEAC medical entomology laboratory since more than 20 years.

Susceptibility tests were carried out according to the WHO standard protocol for adult mosquitoes using insecticide discriminating dosage on filter papers (50). Insecticide impregnated filter paper sheets (Whatman N°1, 12 cm x15 cm) were purchased from the WHO reference center at the Vector Control Research Unit, University Sains Malaysia. These papers were impregnated with discriminating dosages of insecticides: 0.05% deltamethrin, 0.75% permethrin, 4% DDT and 0.1% bendiocarb. The preparation of stock solution for PBO synergist and impregnation on filter papers (12 cm × 15 cm) were performed by our research unit, in the LRP/OCEAC.

Two types of susceptibility test were performed; test with synergist (PBO) and test without synergist. Tests with synergist

were carried out to assess possible implication of monoxygenase-based metabolic resistance (P450); for this purpose, mosquitoes were exposed for 1 h to 4% piperonyl butoxide (PBO) synergist, prior to exposure to insecticides. All the tests were conducted under ambient room temperature (25 ± 3°C) and relative humidity (70 ± 10%). For each test, four batches of 20–25 mosquitoes were exposed to insecticide impregnated paper; and one-two batches of 20–25 mosquitoes used as control were exposed to papers impregnated with silicon oil, i.e., without insecticide. During exposure to insecticides, the number of mosquitoes knocked down was recorded at 5-minute intervals. After 1 h exposure, mosquitoes were transferred to holding tubes and then fed with a 10% sugar solution. The mortality rates were determined 24h post exposure.

Dead, survivor and control mosquitoes were individually kept in Eppendorf tubes with silica gel and stored at −20°C for molecular analyses.

The resistance status was evaluated according to the WHO criteria (50), which classify mortality rates less than 90% as indicative of resistance and those greater than 98% as indicative of susceptibility. Mortality rates between 90–98% suggest the possibility of resistance that needs to be verified.

Kdr L995F/S Genotyping

Two alleles at the kdr 995 locus were genotyped in mosquitoes that have survived exposure to insecticides and identified down to species as described by Santolamazza et al. (49). Their total DNA was analyzed to detect the kdr L995F/S mutations using hot oligonucleotide ligation assay (HOLA) as described by Lynd et al. (51). Extracted DNA was used for *kdr* status characterization using PCR method (52) then, the genotypes were determined by a hot ligation reaction with detector and reporter oligonucleotides. The kdr L995F/S genotypes were determined using a colorimetric test on microplates previously treated with streptavidin (51).

Statistical Analysis

The Human Biting Rates, the Infection Rates (IR) and the Entomological Inoculation Rates (EIR) were calculated for both retrospective and prospective data. The proportions of each species before and following the urbanization process were compared using theChi square test for equality of proportions. The average HBR, IR, and EIR at 95% confidence interval were compared using the ANOVA test. These analyses were done on R 3.5.0 software (R Development Core Team, Vienna, Austria, 2018).

The knockdown times for 50% and 95% of tested mosquitoes (kdt_{50} and kdt_{95}) were estimated using a log-time probit model (53), performed with WIN DL (version 2.0, 1999) software. The kdt_{50} recorded from field-collected mosquitoes were compared with that of the Kisumu reference susceptible strain of *An. gambiae* by estimates of kdt_{50} Ratios (kdt_{50}R). kdt_{50} Ratio >2 fold compared with the Kisumu reference susceptible mosquito strain indicates a significant increase of knock-down times (54). The reversion of knockdown times by PBO was estimated as described by Thomas et al. (55) using the formula Reversion of Knockdown time = (1–(kdt_{50} PBO + insecticide/kdt_{50} insecticide)) x 100.

The mortality rate in the control sub sample (i.e., exposed to silicon oil impregnated papers) was 0-3%, therefore, Abbot's correction was not necessary during data analysis. The mortality rates of mosquitoes tested with insecticides alone were compared to that of specimens pre-exposed to PBO by means of a Chi square Mantel Haenszel test.Allelic and genotypic frequencies at the kdr 995 locus were calculated using Genepop online Version 4.5.1 (56).

Ethical Approval and Consent to Participate

The study was conducted under the ethical clearance no. 2016/01/685/CE/CNERSH/SP delivered by the Cameroon National Ethics (CNE) Committee for Research on Human Health. All volunteers participating in human landing catches signed a written informed consent form indicating their willingness to take part in the study. They also received free malaria prophylaxis.

RESULTS

Composition and Abundance of Anopheline Fauna

A total of 5,341 adult female anophelines were captured in Simbock during the two study periods, including 5,240 by 316 volunteers during the retrospective period (2000–2006) (12-34.5 person–night/month), and 101 by 42 volunteers during the prospective period (2014-2016) (12-18 person–night/month). The distribution of anopheline species in both study periods is shown in **Table 2**.

During the retrospective period, nine mosquito species/complex species were morphologically identified among the collected anopheline samples, namely *An. gambiae* s.l., *An. moucheti*, *An. funestus*, *An. nili*, *An. paludis*, *An. ziemanni*, *An. coustani*, *An. namibiensis* and *An. hancocki*. However, during the prospective period, only three anopheline species/complex species were identified; these include, *An. gambiae* s.l., *An. funestus* and *An. paludis*.

Between years 2000 and 2006 (retrospective period), *An. moucheti* was the major species (49%), followed by *An. funestus* (24.6%), *An. nili* (13.5%) and *An. gambiae* s.l. (12%); the six remaining species were represented at less than 3% each. By contrast from 2014 to 2016 (prospective period), *An. moucheti* and *An. nili* were not found in the collected mosquito samples; *An. funestus* remained the second most abundant species, with a non-significant increase in proportion from 24.6 in 2000-2006 to 31.6 in 2014-2016 (p= 0.12).The proportions of *An. gambiae* s.l. rather increased from 12% in 2000-2006 to 67% in 2014-2016 periods (p <0.0001), setting up this species complex as the major malaria vectors in Simbock.

Molecular identification carried out on 705 anophelines morphologically identified as belonging to the *An. gambiae* s.l. complex revealed the presence of two sibling species, *An. gambiae* and *An. coluzzii*. In general, the proportion of *An. coluzzii* was higher (> 85%) than that of *An. gambiae* (<15%),

TABLE 2 | Distribution of the anopheline species/species complex (A) and the *An. gambiae* s.l. sibling species in Simbock (B).

	2000-2006 N (%)	2014-2016 N (%)	P value
(A) Distribution of Anopheline species/species complex			
An. gambiae s.l.	637 (12.1)	68 (67.3)	<0,0001
An. moucheti	2566 (49)	0	<0.0001
An. funestus	1287 (24,6)	32 (31,6)	0,1266
An. nili	709 (13.5)	0	0.0001
An. paludis	13 (0.2)	1 (1)	0.6439
An. ziemanni	20 (0.3)	0	1
An. coustani	1 (0.02)	0	1
A. namibiensis	1 (0.02)	0	1
A. hancocki	6 (0.05)	0	1
$Total_1$	**5240**	**101**	
(B) Distribution of *An. gambiae* s.l. sibling species			
An. gambiae	52 (8.2)	10 (14.2)	0,5503
An. coluzzii	585 (91.8)	58 (85.8)	0,1231
$Total_2$	**637**	**68**	

N, number of mosquitoes collected per species; (%), percentage; p, value of the comparison of species proportion between the two study periods (signification level at 5%).

and no significant difference of the distribution of these two species was observed between the two study periods ($p > 0.05$).

Plasmodium falciparum Infections and Entomological Inoculation Rates

Overall, 3,619 female anophelines were analyzed by ELISA CSP for *P. falciparum* infection detection, including 3,518 collected in 2000-2006 and 101 in 2014-2016. Data on *P. falciparum* infection and entomological inoculation rates of anopheline species in the both study periods are shown in **Table 3**. The mean infection rates decreased from 2.1% in 2000-2006 to 1% in 2014-2016, though the difference was not significant (P=0.4). Likewise, the entomological inoculation rate decreased from 0.34 ib/m/n to 0.02 ib/m/n (P<0.0001) between the two study periods. Between years 2000 and 2006, four species were responsible for *P. falciparum* transmission, these include *An. moucheti* (IR=1.5, EIR=0.12 ib/m/n), *An. funestus* (IR=2.9, EIR=0.11 ib/m/n), *An. gambiae* s.l. (IR=3.9, EIR=0.07 ib/m/n) and *An. nili* (IR=1.8, EIR=0.03 ib/m/n). These species were involved in plasmodial transmission both indoors and outdoors, with EIRs ranging from 0.01 pi/h/n to 0.1 pi/h/n. Between years 2014 and 2016, no infective bite was recorded in *An. funestus* (in- and outdoors) as well as *An. gambiae* s.l. collected indoors. Only *An. gambiae* s.l. collected outdoors was found infected by *P. falciparum* (IR=1.5%, EIR=0.02 ib/m/n). There was no significant difference of outdoor EIRs of *An. gambiae* s.l. between the two study periods (p=0.6).

Susceptibility of *An. gambiae* s.l. to Insecticides

A total of 21 susceptibility tests were performed between 2014 and 2017, including 6 tests with mosquitoes of the reference susceptible strain Kisumu and 15 tests with wild mosquito samples. Among these, sixteen tests were conducted with insecticides only and five tests with insecticide-synergist combination whereby mosquitoes were exposed to 4% PBO synergist prior to pyrethroid insecticide susceptibility tests.

Knockdown and Mortality Rates of *Anopheles gambiae* s.l.

The Kisumu *An. gambiae* reference strain was found fully susceptible to deltamethrin, permethrin, DDT and bendiocarb, with 100% mortality rates. The kdt_{50} were less than 10 minutes to

TABLE 3 | Variations of malaria infection rates and entomological inoculation rates indoor and outdoor.

Species	Indoor							Outdoor						
	2000-2006			2014-2016				2000-2006			2014-2016			
	N	IR	EIR	N	IR	EIR	P value	N	IR	EIR	N	IR	EIR	P value
A. moucheti	1764	1.3	0.1	0	ND	ND	ND	748	2.2	0.1	0	ND	ND	ND
A. funestus	906	2.4	0.1	21	0	0	ND	343	4.7	0.1	11	0	0	ND
A. gambiae s.l.	379	3.6	0.1	35	0	0	ND	167	4.8	0.05	33	3.0	0.04	0.6
A. nili	512	2.0	0.06	0	ND	ND	ND	176	1.4	0.01	0	ND	ND	ND
Total	3561	2.3	0.3	56	0	0	ND	1434	3.3	0.06	44	1.5	0.02	0.5

ND, Notdetermined; IR, infection rate; EIR, entomological inoculation rate; N, number of mosquitoes analyzed.

deltamethrin and permethrin with or without PBO and 19.1 minutes to DDT. The knockdown times for 95% mosquitoes (kdt_{95}) varied from 14 to 31 minutes (**Table 4**).

The knockdown times for 50% (kdt_{50}) and 95% (kdt_{95}) mosquitoes tested with insecticides alone or in combination with PBO are given in **Table 5**, and mortality rates in **Figure 2**.

The wild *An. gambiae* s.l. samples regularly exhibited resistance to pyrethroids and DDT. Between 2014 and 2017, there was a significant increase of kdt_{50} to deltamethrin from 37 minutes to 58 minutes, with an upsurge of kdt_{50} ratios compared to the Kisumu reference strain from 3.9 in 2014 to 6.1 in 2017. The kdt_{50} to permethrin and DDT were always above 60 minutes (**Table 5**).

The mortality rates were 50-90% to deltamethrin, less than 40% to permethrin and less than 10% to DDT, with some variations from one year to another. However, this tested mosquito samples were fully susceptibility to bendiocarb, with mortality rate of 100% at each of the test period.

PBO Effect on *An. gambiae* s.l. Knockdown Times and Mortality Rates

Pre-exposure of wild mosquitoes to PBO resulted in 35% reversion of knockdown times with deltamethrin. However, the recorded kdt_{50} with permethrin remained greater than 60 minutes.

Mortality rates due to deltamethrin in combination with PBO synergist increased from 90% in 2015 to 100% in 2016, while the mortality rate recorded with permethrin remained lower than 30%. Tests with deltamethrin and permethrin combined with PBO were not performed in 2017 because of low sample size.

Kdr L995F/S Genotypic and Allelic Frequencies

A total of 80 mosquitoes randomly selected among those which survived exposure to pyrethroids, were genotyped for kdr 995F/S mutations. These mosquito samples were composed of *An. coluzzii* (N=72; 90%) and *An. gambiae* (N=8; 10%). The two kdr L995F and L995S alleles were found in *An. coluzzii*, while only the L995F allele was observed in *An. gambiae* (**Table 6**). Furthermore, there was a high genotypic diversity, with all possible genotypes being represented both in *An. gambiae* and *An. coluzzii*.

In the *An. gambiae* subsample, the kdr L995F allele was recorded at 70% frequency. The predominant genotype was L995F/L995F homozygote resistant (50%), followed by L995L/L995F heterozygote (37.5%) and L995L/L995L homozygote susceptible (12.5%).

In the *An. coluzzii* subsample, in addition to the kdr L995F allele found at a 60% frequency, the kdr L995S allele was also found, although at a heterozygote state with L995F in only one mosquito specimen (1% allelic frequency). The heterozygote

TABLE 4 | Knockdown times of the Kisumu *Anopheles gambiae* s.s. reference strain to insecticides with or without PBO synergist.

Insecticide	N	Kdt_{50} [CI50] (min)	Kdt_{95} [CI95] (min)	Status
Test without synergist				
0.05% Deltamethrin	100	9.5 [8.4-10.8]	17.3 [15.7-19.4]	S
0.75% Permethrin	100	8.8 [7.3-11.1]	14.0 [12.6-17.8]	S
4% DDT	100	19.1 [17.8-21.1]	31.2 [28.5-33.1]	S
0.1% Bendiocarb	100	–	–	S
Test with synergist				
0.05% Deltamethrin+PBO	100	9.2 [7.8-10.8]	18.4 [17.2-20.4]	S
0.75% Permethrin+PBO	100	9.7 [7.8-11.5]	17.2 [16.1-20.6]	S

N, sample size; kdt_{50} and kdt_{95}: knockdown times for 50% and 95% of the tested samples; S, susceptible; PBO, piperonyl butoxide.

TABLE 5 | Knockdown times and resistant status of wild *Anopheles gambiae* s.l. samples to insecticides with or without PBO synergist.

Test configuration	Insecticide DC	Year	N	Kdt_{50} [CI50] (min)	Kdt_{95} [CI95] (min)	Kdt_{50} R	Status
Tests without PBO	0.05% Deltamethrin	2014	46	37.2 [34.5-40.6]	>60	3.9	R
		2015	98	53.9 [51.1-57.6]	>60	5.6	R
		2016	113	47.1 [42.8-54.1]	>60	5	R
		2017	89	58.2 [54.0-64.6]	>60	6.1	R
	0.75% Permethrin	2015	45	>60	>60	ND	R
		2016	100	>60	>60	ND	R
		2017	61	>60	>60	ND	R
	4% DDT	2014	41	>60	>60	ND	R
		2016	103	>60	>60	ND	R
	0.1% Bendiocarb	2015	92	–	–	–	S
		2016	70	–	–	–	S
		2017	64	–	–	–	S
Tests with PBO	0.05% Deltamethrin	2015	84	35.0 [29.7-40.5]	> 60	35*	PR
		2016	100	30.9 [27.4-33.8]	53.3 [47.2-66.0]	35*	S
	0.75% Permethrin	2016	76	>60	>60	ND	R

*PBO, piperonyl butoxide; Insecticide DC, insecticide diagnostic concentration; N, sample size; kdt_{50} and kdt_{95}, knockdown times for 50% and 95% of the tested samples; kdt_{50}R, ratio kdt_{50} wild mosquito sample/kdt_{50} Kisumu strain; min, minutes; * knock down time reversion rate; ND, not determined; S, susceptible; R, resistant; PR, probable resistant.*

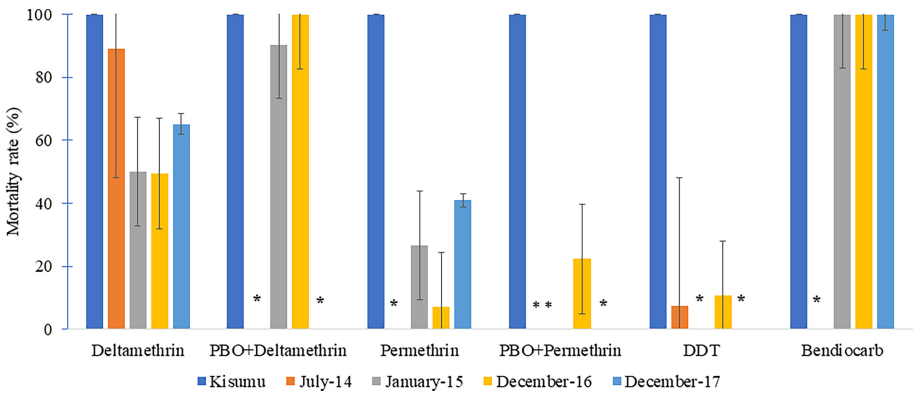

FIGURE 2 | Mortality rates of *An. gambiae* s.l. of the Kisumu strain and field samples from Simbock to insecticides between 2014 and 2017. * Mortality rates not determined because the assay was not performed.

L995L/L995F was the most represented (46%) genotype, followed by the homozygote resistant L995F/L995F (39%) and the homozygote susceptible L995L/L995L (14%).

The kdr L995F allelic frequencies were similar in both *An. gambiae* and *An. coluzzii* surviving subsamples (p= 0.5).

DISCUSSION

The main objective of this work was to assess malaria transmission and vector susceptibility to insecticides in the Simbock neighborhood, following the environmental changes resulting from recent urbanization. Previous studies conducted in this locality between 1999 and 2009 have revealed the presence of six anopheline species, namely *An. moucheti, An. funestus, An.gambiae* s.l., *An. nili, An. namibiensis* and *An. obscurus*; with *Plasmodium* parasite transmission mainly carried out by *An. moucheti, An. funestus* (0.28-0.22 ib/m/n EIR), followed by *An. gambiae* s.l. and *An. nili* (0.13-0.06 ib/m/n EIR) (38, 39, 41). Data from the current study revealed that after a decade in which the urbanization process was accelerated in Simbock, the species distribution of anophelines has changed. Furthermore, *An. gambiae* s.l. resistance to pyrethroid insecticides is reported in this area for the first time. Indeed, we observed a decrease in the number of anopheline species from nine species recorded between years 2000 and 2006 to three species between 2014 and 2016. Furthermore, we have highlighted the persistence of *Plasmodium sp.* transmission in Simbock (0.02 ib/m/n) by *An. gambiae* s.l., which is now responsible for urban malaria in Simbock, as previously reported by Antonio et al. (39).

One limitation of this study is that, the number of entomological surveys conducted during the retrospective period (14 surveys during the four seasons of the year) was greater than that conducted during the prospective period (3 surveys during 3 seasons). Furthermore, during the prospective period, no survey was conducted during the short dry season. The small number of field surveys, the seasonal variations and bias in sampling sites could influence the outcomes of this study. It has been demonstrated that malaria transmission has a significant association with meteorological variables such as temperature, rainfall and humidity which play a major role in the life cycle of the malaria vectors and have been associated with their dynamics (57). More specifically, a recent study conducted in Cameroon in 2021 has revealed high anopheline densities and *Plasmodium* sp. parasite transmission during the short rainy season compared to the long rainy season (58). Therefore, the mosquito collections made during the two rainy seasons as well as the long dry season could allow to highlight the variations of the mosquito diversity associated with the urbanization process in Simbock. Indeed, the species composition and the infectivity of the anopheline fauna obtained during this prospective period are in agreement with previous findings in other neighborhoods of the Yaoundé city (22, 58). During the prospective period of the current study, the profile of malaria transmission in Simbock became that of urban malaria with increased densities of anthropophilic vectors such as *An. gambiae* and *An. funestus*

TABLE 6 | Kdr L995F/S genotypic and allelic frequencies in wild *An. gambiae* and *An. coluzzii* samples which survived exposure to pyrethroid insecticides.

Species	N	(%)	kdr genotypic frequency				kdr allelic frequency		
			SS	RwS	RwRw	RwRe	S	Rw	Re
A. gambiae	8	10	0.125	0.375	0.50	0	0.3	0.7	0
A. coluzzii	72	90	0.14	0.46	0.39	0.01	0.3	0.6	0.1

N, number of mosquito tested; SS, homozygote-susceptible 995L/L; RwS, heterozygote 995L/F; RwRw, homozygote 995F/F; RwRe, heterozygote 995F/S; S, susceptible allele L995L; Rw, resistant allele kdr L995F; Re, resistant allele kdr L995S.

and high feeding likelihood due to increased availability of human hosts following the demographic explosion.

Despite the small sample sizes used for EIR estimates, the presence of *An. gambiae* the major malaria vector in urban settings, is strongly suggestive of local *Plasmodium sp.* transmission (59). Furthermore, mosquito dispersal is much more limited in urban areas due to the higher housing density (60), causing urban malaria transmission to be highly focal (61). The destruction of vegetation due to land clearing along the Mefou River that crosses Simbock and deforestation may have led the disappearance of forest-dependent species such as *An. moucheti*, *An. nili*, *An. namibiensis* and *An. hancocki* which thrive in roosts with erect vegetation. The current predominance of *An. gambiae* s.l. in the Simbock neighborhood is in agreement with Ndoumbe et al. (62), who highlighted the role of species of the *An. gambiae* complex in malaria transmission across the most urban settings in Cameroon. The emergence of *An. gambiae* in Simbock could be explained by the migration of this species from the central neighborhoods to the periphery, attracted by the strong demographic pressure. Furthermore, *An. gambiae* is demonstrating a worrying trend of adaptation to polluted waters in urban environments (19). In recent years, this species has been found breeding in highly polluted water sources in Cote d'Ivoire (21) and Cameroon (22). In addition to *An. gambiae* s.l., *An. funestus* also appeared as an emerging species in the newly anthropized environment of Simbock, in agreement with Antonio et al. (40) and Munga et al. (63), despite the low abundance of these two species during the prospective period compared to the retrospective period. Ponds that were already present at Simbock during the retrospective period and that became poorly maintained during the prospective period, may have served as permanent or semi-permanent breeding sites for *An. funestus.* The low abundance of anopheline species during the prospective period could be partly due to many factors such as house construction with concrete floors and walls, ceiling under the roof and screened windows during the prospective period, insecticide-treated net (ITN) use, and other factors that influence human–vector contact such as environmental sanitation (64). Moreover, higher socioeconomic status through better education, higher exposure to TV and radio prevention campaigns, and increased ability to afford prevention methods, all contribute to increased vigilance of populations with regard to malaria vectors (29, 30, 65). Therefore, the intensification of vector control interventions could partly explain the lower anopheline abundance in Simbock during the prospective period. Indeed, the National Malaria control Programme has conducted two large-scale distributions of LLINs in 2011 and 2015, increasing the net ownership from 65,6% to 76,6% (66, 67). Moreover, Bamou et al. (68) showed that changes in mosquito bionomics and malaria transmission patterns were consistent with the influence of treated net use and the lack of bed net usage increased the risk of malaria infection 2.2-fold.

The distribution of sibling species of the *An. gambiae* s.l. complex remained unchanged between the two study periods, with *An. coluzzii* being the most represented species. The predominance of *An. coluzzii* in Simbock could be due to the presence of permanent breeding sites both before and after urbanization; which is consistent with previous studies (69, 70). It should be recalled that *An. coluzzii* is a species which colonizes urban environments (22, 26, 69); furthermore, this species is very likely to carry insecticide resistance genes, in particular the kdr L995F gene (34). Furthermore, the fact that this major malaria vector usually exhibits similar biting behavior indoors and outdoors, underlines the risk of contracting malaria both inside and outside dwellings. These data highlight the need for complementary vector control interventions on top of LLINs to control outdoors malaria transmission. Based on the fact that *An. coluzzii* develops in permanent roosts, larviciding operations which are under experimentation in the Yaoundé city (71) could be considered a potential complementary malaria vector control intervention in Simbock.

The insecticide susceptibility tests of *An. gambiae* s.l. carried out between 2014 and 2017 showed that *An. gambiae* s.l. from Simbock has developed resistance to DDT and pyrethroid insecticides, while remaining susceptible to bendiocarb. The frequencies of pyrethroid resistance recorded in Simbock are similar to those recently reported in other neighborhoods of Yaoundé, i.e., Nkolondom, Ekié and Nkolbisson, where market gardening is practiced throughout the year with uncontrolled use of insecticides (72). In the specific case of Simbock, theobserved resistance profile could be explained by the migration of *An. gambiae* species carrying the resistance alleles, from the central neighborhoods where market gardening is practiced to the periphery. Similar observations have already been made in other African countries (73, 74). To explore the role of target site mutations in the observed pyrethroid resistance, we conducted a preliminary screening of kdr 995F/L allele in mosquitoes which survived exposure to insecticides. Indeed, Donnelly et al. (75), asserted that the kdr L995F/F resistant homozygote genotype is correlated with DDT- and pyrethroid-resistance phenotype. Also, a strong correlation between high frequencies of kdr L995F allele and pyrethroid and DDT resistance was reported in several studies (76–78). Since the first report of resistance to insecticide in *A. gambiae* s.l. population in Cameroon (79, 80), the level of pyrethroid and DDT resistance has been increasing, alongside with the frequency of the L995F *kdr* allele (34, 81, 82).The kdr L995F allele was reported in this study at 70% frequency in *An. gambiae* and 62% in *An. coluzzii*. These high allelic frequencies of the kdr L995F were associated with high frequencies of homozygotes resistant and heterozygotes genotypes in both *An. gambiae* and *An. coluzzii.* These results are consistent with those reported in pyrethroid resistant populations of *An. gambiae* s.l. from others cities of Cameroon (54–56) and in West Africa (18, 52, 54, 83, 84). The resistant L995S allele was also found in *An. coluzzii* from Simbock, although at a very low frequency (1%). The scarcity of this allele in Cameroon has been reported in previous studies (80, 81). Santolamazza et al. (49) pointed out that the L995S mutation has a much more restricted geographic distribution in East Africa unlike the L995F mutation which is widespread in West and Central Africa. In the same vein, Nwane

et al. (82) suggest that the L995S allele disperses from East to West and the L995F allele in the opposite direction. Although found at a low frequency, the kdr L995S mutation is already installed and continues to spread throughout Cameroon (85). Therefore, in order to assess the real frequencies of kdr L995F/S alleles in natural populations of *An. gambiae* and *An. coluzzii* from Simbock, further studies with representative sample sizes of randomly selected mosquitoes not exposed to insecticides are needed.

In the other hand, the presence of the susceptible homozygous L995L/L995L genotype in pyrethroid resistant mosquitoes suggest the involvement of alternative resistance mechanisms, or of some cofactors not yet identified (86). As such, we found an increase of mortality rates and 35% knockdown times reversion in mosquitoes pre-exposed to PBO synergist, suggesting the involvement of P450s' detoxifying enzymes in tested *An. gambiae* s.l. resistant to deltamethrin. These results are consistent with previous studies conducted in the equatorial areas of Cameroon (32, 80, 82, 87). The lack of inhibitory effect of PBO on permethrin resistance suggests less involvement of P450s' in the resistance to this insecticide. Such resistance could then be essentially linked to the "kdr" type mutations.

Given the fact that only survivor mosquitoes were genotyped for the kdr mutations and that very few susceptibility tests were carried out with PBO, a comprehensive study on the evolution of both kdr and metabolic resistance mechanisms is needed to assess the intensity and evolution of *An. gambiae* s.l. resistance to pyrethroid insecticides in Simbock. Nevertheless, tested mosquito samples remained full susceptible to bendiocarb; therefore, IRS with this insecticide may be advocated to complement LLINs in this area, in addition to bio larvicides.

CONCLUSION

This study highlights the occurrence of urban malaria transmission in Simbock, and describes for the first time, the profile of multiple pyrethroid resistance mechanisms in the local *An. gambiae* s.l. populations. It is therefore necessary to set up resistance management strategies based on an alternative vector control intervention, e.g., PBO LLINs, larviciding, housing IRS with bendiocarb.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Conceptualization, JE and LM. Data curation, LM, JT and PA-A. Investigation and Methodology, LM, SM,WE and JT. Software, BF. Data analysis, LM, BF. Project administration, PA-A. Resources, PA-A. Supervision, AF and JE. Validation, PA-A, AF and JE. Writing—original draft, LM. Writing—review and editing, LM, WE, SM, MP, LA, HO, PA-A, PN, AF and JE. Supervision, JE. All authors contributed to the article and approved the submitted version.

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