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Tchoffo Fobasso Roméo Martial
Laboratory of Animal Biology and Physiology, University of Douala, Douala, Cameroon Po. BOX 24157 Douala, Cameroon

Eyebe Serge
Faculty of Medicine and Biomédical sciences, University of Yaounde I, P.O. Box 812, Yaounde, Cameroon

Akono Tonga Patrick
Laboratory of Animal Biology and Physiology, University of Douala, Douala, Cameroon Po. BOX 24157 Douala, Cameroon

Yetchom Fondjo Jeanne Agripine
Laboratory of Animal Biology and Physiology, University of Douala, Douala, Cameroon Po. BOX 24157 Douala, Cameroon

Tagne Darus
Laboratory of Animal Biology and Physiology, University of Douala, Douala, Cameroon Po. BOX 24157 Douala, Cameroon

Atangana Bita Gael
Faculty of Science, University of Yaoundé 1, Po Box 812, Yaoundé-Cameroon

Ngaha Rachel
Laboratory of Animal Biology and Physiology, University of Douala, Douala, Cameroon Po. BOX 24157 Douala, Cameroon

Mamia Grace Florentine
Laboratory of Animal Biology and Physiology, University of Douala, Douala, Cameroon Po. BOX 24157 Douala, Cameroon

Ngangue Siewe Idris
Centre for Research in Infectious Diseases, Yaounde Cameroon

Lehman Léopold Gustave
Laboratory of Animal Biology and Physiology, University of Douala, Douala, Cameroon Po. BOX 24157 Douala, Cameroon

MBIDA Jean Arthur
Laboratory of Animal Biology and Physiology, University of Douala, Douala, Cameroon Po. BOX 24157 Douala, Cameroon

Corresponding Author:
MBIDA Jean Arthur
Laboratory of Animal Biology and Physiology, University of Douala, Douala, Cameroon Po. BOX 24157 Douala, Cameroon

Anopheles KDR mutation threatens the operational effectiveness of malaria vector control strategy in the health district of Ngaoundere, savannah area in north Cameroon

Tchoffo Fobasso Roméo Martial, Eyebe Serge, Akono Tonga Patrick, Yetchom Fondjo Jeanne Agripine, Tagne Darus, Atangana Bita Gael, Ngaha Rachel, Mamia Grace Florentine, Ngangue Siewe Idris, Lehman Léopold Gustave and MBIDA Jean Arthur

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Abstract
Malaria prevention in Cameroon mainly relies on insecticide treated bet nets mass use. Its success depends on vectors sensitivity to insecticides used and population's involvement. This study aimed at determining the operational effectiveness of treated bet nets and find ways to improve the vector control strategy in Ngaoundere and Tchabal. Knowledge, attitudes and practices of populations toward malaria were assess. The susceptibility of local populations of *Anopheles gambiae* to deltamethrin and permethrin, the effect of synergist (PBO) and mosquito treated nets efficacy were determined using WHO protocols. Mosquitoes were genotyped for kdr L1014 mutations using Hot Oligonucleotide Ligation Assay. Mosquito displayed insecticide resistance with variable knockdown and mortality rates. Synergist (PBO) increased mortality but not enough to break their resistance. We found 78.33% and 93.33% Kdr resistant allele and in Tchabba and Ngaoundere respectively. New mosquito nets generation with PBO should be combined with other control methods to curb resistance.

Keywords: Malaria, vector control, LLIN, operational effectiveness

Introduction
Despite several years of control efforts, malaria remains a major public health problem world-wide, especially in sub-saharan Africa where 92% of reported cases are found [1]. According to the World Health Organization (2019), an estimated 219 million cases of malaria and 405,000 deaths occurred worldwide in 2018. The high transmission level observed in Sub-saharan Africa is often explained by eco-climatic conditions which are found favorable for malaria vector development [2]. In Cameroon, though several strategies have been developed to fight against malaria, efforts are currently channeled towards vector control strategies [3]. Thus, mass distribution campaigns of Long Lasting Insecticidal Nets (LLINs) are regularly carried out throughout the country with special focus on people at risk [4]. These nets are supported by a high use of insecticides by households. According to the estimates of a recent study, the mass use of LLINs observed in sub-Saharan Africa had a successful impact because it saves around 7 million people between 2000 and 2015 [5]. Unfortunately, entomological surveys recently conducted in Cameroon revealed wide distribution of the pyrethroids resistance [6, 7] which are insecticides recommended for nets impregnation [8]. Thus, this resistance is becoming a real threat for vector control programs as they are mainly based on insecticides [9]. However, the resistant status of vectors and the effect of the insecticide present on LLINs are still unknown in Vina division, although these data are necessary to improve the vector control strategy. Also, the human factor must be taken into account in the development of the vector control strategies. Many studies showed that the knowledge, attitudes and practices of populations significantly influence the effectiveness of control program [10, 11]. In order to develop a comprehensible and effective vector control plan, it is therefore useful to have permanent information on the operational effectiveness concerning the tools used. This study carried out in the town Ngaoundere and the village Tchabbal aimed to determine the susceptibility of *A. gambiae* local populations to pyrethroids; the efficacy of

LLIN against these vectors; and assess the knowledge and practices residents on malaria prevention measures.

Methods

Study site

The survey was conducted in two localities within Vina Division, Adamaoua Region of Cameroon, in urban area in Ngaoundere city (7°19'N, 13°35'E) and rural area in Chabbal village (7°31'57.0N, 13°33'30,0E) from november 2019 to january 2020. The climate is of sudano-guinean highland type with annual rainfalls of 1600 to 1800 mm distributed over 7 to 8 months and the relative humidity is between 40-60%. The annual average temperature is 23°C [10, 12]. The rainy season lasts 8-9 months, with 6 to 8 months of heavy rainfall (from March to October); the dry season lasts at least 3 to 4 months (from November to Febuary). According to Etang (2003) [13], the main activities of the populations are cattle breading, trade and small-scale agriculture. The territory is sparsely occupied with 18 inhabitants/km² and mainly populated by Fulani groups. Malaria transmission is seasonal in this area with *Anopheles gambiae s.l.* as the main vector [14].

Household survey for population involvement, LLINs couverage, utilisation and integrity

In Ngaoundere, the survey was conducted in 10 neighborhoods (plateau, Beka-Océré, Yarban, Sabongarie, 12 poteaux, Dang, Bini, Baladji II, Jolie soir, Burkina), with 50 households visited in each. In the Chabbal village, 276 households were randomly visited due to the structure of the village. Household head, his spouse or any adult who consented to the study, were interviewed using a structured pre-tested questionnaire to determine their level of knowledge, attitudes and practices toward malaria. Many indicators were evaluated:

- Knowledge of malaria (signs and symptoms),
- Malaria vectors,
- Different prevention methods used,
- Management of malaria cases within households,
- Possession and utilization of ITNs [15],
- Physical integrity of nets used in households.

The physical integrity and utilization of nets were appreciated by direct observations. A net was considered to be used when it was hanged, and its owner declared having used it during the last night before the survey. Regarding physical integrity, each net was scanned on all sides checked whether or not there were any holes or damage.

LLINs Sampling and Storage

Eight LLINs were randomly collected from eight households in Ngaoundere (4 households) and Chabbal (4 households). At least 150m step between the households was relevant to ensure that bed net collection points were representative of the study areas. All the collected mosquito nets were brought to a local laboratory for sampling. From each mosquito net, 25 cm² pieces of netting were removed (three piece per side of the net) according to the WHO protocol [16], wrapped in aluminum foil, labeled and stored for subsequent bioassays with field and laboratory mosquito samples.

Mosquito collection and rearing

Immature stages of anopheles were collected in breeding sites. The collected individuals were brought to the insectarium and raised to the adult stage. After emergence, adult mosquitoes were morphologically identified using the determination key of Gillies and Coetze [17]. Two to five days old *An. gambiae* complex mosquitoes were used for bio-efficacy tests. The susceptible *An. Gambiae* kisumu strain was used as controls for bio-efficacy tests.

Insecticide susceptibility test

Susceptibility of adult mosquitoes to insecticides were carried out using WHO standard procedures with 0.75% permethrin and 0.05% deltamethrin like diagnostic concentrations. For each test, four batches of 20-25 non blood fed, 2 to 4 days old females were exposed to a discriminating doses of insecticide and two batches of 20 to 25 non blood fed mosquitoes were used as control. During exposure to insecticides, the number of mosquitoes knocked-down was recorded at 5-min intervals. After 1-hour exposure to insecticide-impregnated papers or control papers, the number of knockdown mosquitoes was recorded, mosquitoes were transferred to holding tubes and provided with cotton pads soaked with 10% sugar solution. The mortality rates were recorded after 24-hours. Tests were performed under ambient room temperature (25–28 °C) and relative humidity of 70–80%. To assess the implication of metabolic resistance, additional tests were carried out with four batches of 20–25 non blood fed mosquitoes exposed for 1 h to 4% piperonylbutoxide (PBO) synergist, prior to exposure to each insecticide.

Susceptibility tests were performed with the Kisumu susceptible strain of *An. gambiae* s.s.as well to confirm the quality of papers.

LLINs bio-efficacy tests

The residual bio-efficacy of LLINs was assessed using the WHO cone test [15]. Four plastic cones were fixed to each piece of netting. Batches of 5-10 non-blood fed, two to four days old female mosquitoes were transferred in each cone for three minutes exposure to net samples and held for 24 hours with access to 10% sugar solution. Twenty to forty mosquitoes (5 mosquitoes x 4 cones) were exposed to each piece of netting and results from the five pieces of each mosquito net were pooled for analysis. Mosquitoes exposed to untreated nets were used as negative control. Bioassays were carried out at 27 ± 2 °C and 75 ± 10% RH. Mosquito knock-down rates were measured 60 minutes post exposure and mortality rates after 24 hours holding period.

Molecular identification and *kdr* L1014 genotyping of tested mosquito

Total DNA of dead and survivor mosquitoes after bioassays, was extracted following Collins *et al.* in 1987 [18]. Molecular identification was performed with each extract using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) as described by Fanello *et al.*, [19]. Alleles at locus 1014 were identified using Hot Oligonucleotide Ligation Assay (HOLA) [20].

Data analysis

Data was saved in a 2016 Microsoft Excel spreadsheet and the graphs were plotted using this spreadsheet. Statistical analysis were performed using Statistical Package for Social Sciences (SPSS) software.

- Coverage rate was calculated according to the principle of a mosquito net for two members of the household [15]:
- Protection of sleeping spaces (beds, mats, mattresses placed on the floor) was determined by dividing the

number of mosquito nets available by the number of sleeping spaces counted within households. Rates of nets used was determined by dividing the number of nets in use by the number of nets available in households. Holes and tears on mosquito nets have been classified into four categories according to their size [21]: Size 1 (0.5 - 2cm); Size 2 (2 - 10cm); Size 3 (10 - 25cm); -Size 4> 25cm. These categories were used to calculate the hole indices (PHI) according to WHO recommendations. PHI = (a x number of holes in T1) + (b x number of holes in T2) + (c x number of holes in T3) + (d x number of holes in T4); with a = 1, b = 23, c = 196 and d = 578. On the basis of calculated IHP, the nets were classified into three categories: good mosquito nets (0 <pHI<64); acceptable mosquito nets (65 <pHI<642); disposable mosquito nets (pHI> 642).

Mosquitoes mortality rates of tested with insecticides alone were compared to that of specimens pre-exposed to PBO by means of a Chi square Mantel Haenszel test. Mortality and knock-down rates of exposed mosquitoes were calculated and analyzed according to WHO criteria. A mosquito net was considered effective when the knockdown rate of exposed mosquitoes was>95% and the mortality rate> 80%.

Results

Socio-demographic characteristics

A total of 776 households were visited, including 500 in Ngaoundere and 276 in Chabbal. The total population recorded in these households was 3,902 peoples, with 22.77% (N = 1,454) of vulnerable persons (pregnant women and children < 5). The average number of people observed per household was 6 (min 1; max 11) and (min 3; max 19) in Ngaoundere and Chabbal.

Malaria Knowledge and home management

Data on population knowledge in relation to symptoms, transmission and case management of malaria are summarized in Table 1.

More than 88% (n=688) of person interviewed knew at least one sign or symptom of malaria and fever was the most cited sign (n = 589; 85.61%).

Concerning malaria transmission: 81.4% (n=407) of persons in Ngaoundéré and 76.45% (211) in Chabbal knew mosquitoes as the main vectors responsible for malaria transmission. Others gave wrong answers as they attributed malaria transmission to various factors (cold weather, consumption of dirty water, and consumption of mangoes).

Concerning malaria prevention: Four malaria preventive methods were reported by households: bed nets use, insecticides use (spray/coil), environmental sanitation and grids installation on doors and windows. Among these, bed nets use was the most cited measure in both study sites (64.40% in Ngaoundere and 80.07% in Chabbal).

Concerning home management of malaria cases: about 40.60% of people in Ngaoundere and 44.56% in Chabbal used hospital facilitations when their family members feel having malaria, while the rest of household used automedication or traditional medicine.

Table 1: indicators on level of knowledge, prevention measures and case management of malaria by households.

Indicators		Ngaoundere (%)	Tchabbal (%)	Both Localities (%)
Symptoms of malaria	Good answer	436(87.20)	252(91.30)	688(88.66)
	False answer	64(12.80)	24(8.70)	88(11.34)
Mode of transmission	Mosquito bites	407(81.4)	211(76.45)	618(79.64)
	Others	93(18.6)	l65 (23.55)	158(20.36)
Prevention measures	Net only	337(67.4)	221(80.7)	558(71.90)
	Insecticides	16(3.20)	1(3.62)	17(2.19)
	Sanitation	12(2.4)	12(4.35)	24(3.09)
	Using nets on windows	11(2.2)	2(0.72)	13(1.67)
	Bet net + insecticides	72(14.40)	8(2.89)	80(10.31)
Home management cases	Nothing	53(10.6)	8(2.89)	61(7.86)
	Hospital	203(40.60)	123(44.56)	326(42.01)
	Self-medication	272(35.05)	97(12.50)	104(13.40)
	Traditional medicine	25(5.0)	56(20.29)	81(10.44)

%= percent;

Mosquito nets coverage and use

A total of 3127 nets was counted in the 776 households visited, i.e. 1998 in Ngaoundere city and 1127 in Chabbal (Table 2). All the nets observed were LLINs from the brands Permanet 2.0, Interceptor, Royal Sentry, Dawa Net and Olyset. The number of households with at least one LLIN was around 90% in the two surveyed areas. The general possession rate was significantly higher in Chabbal (82.37%) than in Ngaoundere (75.81%), (X^2 =34.27; $p<$ 0.001). However, in Ngaoundere and Chabbal, respectively 56.20% and 61.96% of households had the number of nets necessary to protect all members of the household (universal coverage). Regarding the protection of risk persons, the coverage rates were less than 80% in the two sites. The proportion of sleeping spaces (beds, mats, mattresses placed on the floor ...) regularly covered by a LLIN were 73% in Ngaoundere and 89% in Chabbal. Among 3127 LLINs counted in the two sites, 2100 were hanged and regularly used. However, the usage rate was significantly higher in Chabbal than in Ngaoundere ($P<$ 0.03).

Table 2: Coverage and utilization of mosquito nets

Parameters	Ngaoundere	Tchabbal	Both localities
Number of households	500	276	776
Human population in surveyed households	3902	2483	6385
Number of children<5 years (%)	609(15.61)	406(16.35)	1015(15.89)
Pregnant women	243(6.23)	196(7.89)	439(6.87)
Number of sleeping spaces	1998	1129	3127
Number of nets recorded	1479	1023	2502
Mean number of nets per household	2.95	3.70	3.22
Mean number of people per net	2.63	2.42	2.55
Number of households owning at least one net (%)	447(89.40)	254(92.02)	701(90.33)
Number of households owning at least one net for 2 peoples (%)	281(56.2)	171(61.96)	452(58.25)
Number of children< 5 years and pregnant women covered (%)	641(75.23)	478(79.40)	1119(76.96)
Possession rate	75.81	82.37	
Number of sleeping spaces covered	1457(72.92)	1004(88.93)	2461(78.70)
Number of sleeping spaces covered	1194(80.73)	906(88.56)	2461(78.70)

% = percent; <=Lower

Physical integrity of nets

A total of 512 mosquito nets were examined to check their physical integrity, including 239 nets in Ngaoundere and 263 nets in Chabbal. Among these mosquito nets 18.52% (N = 93) had holes. However, the holes index analysis (PHI) shows that more than 95% of examined nets are good and acceptable nets and remain usable (table 3).

Table 3: physical condition of the nets in the city of Ngaoundere and Chabbal village.

Analysis parameters	Ngaoundere (%)	Tchabbal (%)	Total (%)
Number of households	239	263	502
Number of nets with holes	44(17.67)	49(18.63)	93(18.52)
Number of good nets 0≤PHI≤64 (%)	221(78.31)	241(91.63)	462(92.03)
Number of acceptable nets 65≤PHI≤642 (%)	12(4.82)	8(3.04)	20(3.98)
Number of disposables nets PHI>642 (%)	21(8.43)	14(5.32)	35(6.97)

PHI: proportionate hole index, % =percent, <=lower, ≤=less or equal

Status of pyrethroid resistance in *An. gambiae* s. L. populations

Knock-down and mortality rates of mosquitoes tested with insecticides alone or insecticides combined with PBO are given in Table 4.

The Kisumu strain was susceptible to the two insecticides, with 100% of knock-down and 99-100% mortality. On the other hand, the wild mosquitoes samples from Ngaoundere and Tchabbal showed resistance to permerthrine and deltamethrine. Knockdonw and mortality rates were between 62.89-79.80% and 8.14-76.19% respectively.

Prior exposure of wild mosquitoes to PBO synergist resulted in an increase of their knock-down and mortality rates to both insecticides, but the increase displayed was not statistically significant.

Table 4: knockdown and mortality rates of *An. Gambiaes* L. from Ngaoundere and Tchabbal post-exposure to 0.75% permethrin or 0.05% deltamethrin.

Strain	Insecticide	N	KD(%)	Mortality rate (%)
Kisumu	0.05% deltamethrine	98	100	100
	0.75% permethrine	99	100	99.4
Ngaoundere	0.05% deltamethrine	93	76.34	73.14
	0.75% permethrine	86	68.76	8.14
	4%PBO + 0.05% deltamethrine	91	96.70	89.1
	4%PBO +0.75% permethrine	84	95.23	76.19
Tchabbal	0.05% deltamethrine	99	79.80	74.75
	0.75% permethrine	97	62.89	14.94
	4%PBO + 0.05% deltamethrine	87	97.75	89.65
	4%PBO +0.75% permethrine	89	94.38	65.88

PBO: piperonylbutoxide, N: sample size, KD: knockdown, % =percent

Bio-efficacy of Long-lasting Insecticidal Nets (LLINs)

A total of 4912 mosquitoes (2433 *An. gambiae* local strain and 2479 *An. gambiae kisumu*) were exposed on the 120 nets pieces tested (8 nets; 15 pieces / net). The figure 1 shows the knock-down and mortality rates of the different mosquito strains exposed to LLINs. For the Kisumu strain, knockdown rates were higher than 91% while mortality rates ranged between 84 and 97%. Concerning the *An. gambiae* local strain, knock-down rates were between 21 and 79% and the mortality rates were less than 66%.

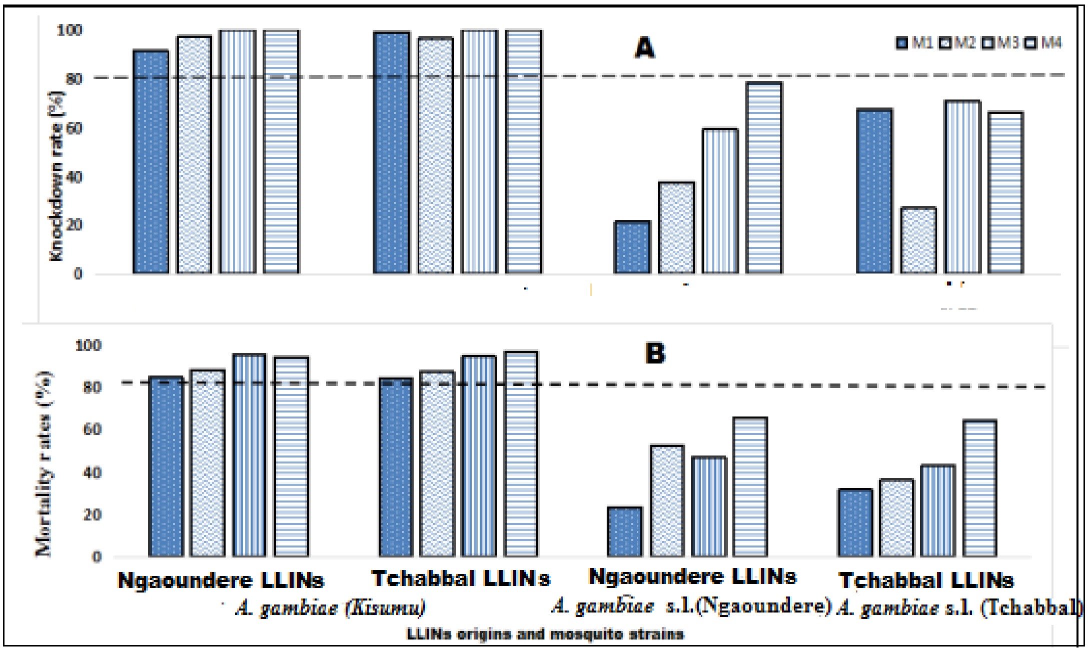

Fig 1: knockdown and mortality rates of *An. Gambiaes* L. from Ngaoundere and Tchabbal post-exposure to 0.75% permethrin or 0.05% deltamethrin.

Mosquito species diversity and frequency of the Kdr allele

A sample of 180 specimens, 90 per locality, were randomly selected among the mosquitoes used for the various tests for species identification. Two species were identified in the city of Ngaoundere, *A. gambiae* and *A. coluzzii*; while in Tchabal, a rural area, three species were identified namely *A. gambiae*, *A. coluzzii* and *A. arabiensis* (Table 5).

Table 5: Distribution of species of the *An. gambiae* complex in Ngaoundéré and Tchabbal.

Species	Ngaoundéré (%)	Tchabbal(%
A. gambia	8(08.89)	12(13.33)
A. coluzzii	82(91.11)	56(62.22)
A. arabiensis	/	22(24.45)

Table 6 shows the frequency of the number of carriers of the L1014 mutation of the Kdr gene among the mosquitoes survivor from the susceptibility tests in each locality. In Tchabbal, the proportion of individuals carrying the resistant allele was 78.33%, ie 71.66% and 6.67% in the homozygous (RR) and Heterozigous state (RS) respectively. In the city of Ngaoundere, the frequency of the resistant allele was 93.33%, i.e. 83% in the homozygous state (RR) and 5% in heterozigous state (RS).

Table 6: Frequency of the number of individual's carriers of the L1014 mutation

Site	N	Mutation *Kdr* L1014F			
		RR	RS	SS	F(R)
Ngaoundéré	60	53	3	4	93.33%
Tchabbal	60	43	4	13	78.33%

N=Sample size, RR=homozygous resistant allele, RS=heterozigous resistant allele, SS= Homozigous sensible allele. *Kdr*=knockdown restitance.

Discussion

The first part of this study aimed to assess the knowledge, attitudes and practices of the populations toward malaria. The majority of interviewed people knew at least one of the symptoms of malaria (88.66%) as well as the transmission mode (79.64%). This could be the population sensibilization consequence carried out by the Ministry of Public Health and their partners through several communication channels [22]. This result is similar to those obtained in several other localities of Cameroon [11, 23].

Over 80% of households used impregnated mosquito nets as the main malaria prevention means. Similar results have already been reported in several localities in the country [2, 24]. These observations showed that the treated mosquito nets mass use promotion made in recent years by the National Malaria Control Program and its partners, positively influenced households' behavior. However, it has been observed that most of these households combined other measures with mosquito nets such as insecticides (sprays/coils), organic material combustion and grids installation on openings. This is an attitude observed in very endemic areas [25], since ITN only protects when you are indoors.

Among households visited, 47.56% practiced self-medication in the event of signs suggestive of fever. Results close to these have already been observed in several other studies [11, 23, 24]. This could be explained by the low knowledge of the risks linked to this practice, but also the remoteness of the hospital center in certain rural areas.

Guiven that the treated mosquito nets used were the main means malaria prevention, the second part of this study aimed to determine the operational efficiency of LLINs through the coverage rate, the sensitivity of mosquitoes to pyrethroids, the bioefficacy and physical integrity of nets used.

Over 90% of households owned at least one LLIN, this is the result of the three mass LLIN distribution campaigns took place throughout Cameroon campaign between 2011 and 2019 [25]. To this is added routine distribution of mosquito nets to pregnant women and children under the age of 5. However, only 58% of households had enough LLINs for universal coverage (one ITN for 2 people). Also, the overall coverage rate (78%) although higher than those obtained by several previously studies carried out in numerous localities [4, 25], remains below the objective set by Cameroon and its partners for an effective fight [3].

The physical barrier offered by LLINs is very important. It prevents human-mosquito contact but depends on the physical integrity of the mosquito nets used [26]. In this study, 18% of LLINs used by households had holes and about 7% of them were in critical state, no longer allowing them to play their role as physical barriers (PHI> 642). This degradation of physical integrity would be due to the duration of use, but also to poor maintenance by some user. It is therefore important to sensitize and train populations in the maintenance of mosquito nets to help maintain the level of coverage.

The present study revealed high resistance of local *A. gambiae* s.l. populations to deltamethrin 0.05% and permethrine 0.75%. Previous studies carried out by Etang *et al*. [13] already suspected resistance to this family of insecticides in Dang (Ngaoundere). Resistance being a dynamic phenomenon, it would have strengthened over time, hence the current result. This resistance of *A. gambiae* to insecticides was further observed with the low bioefficacy of LLINs on local anopheline populations [26].

Molecular analysis of samples collected in the two sites allowed the identification of three species within the *A. gambiae* complex: *A. coluzzii, A. gambiae*, and *A. arabiensis*. The presence of these species has already been reported in the same geographic area [13, 27]. In the Ngaoundere town, only *A. gambiae* and *A. coluzzii* were found, with a strong dominance of *A. coluzzii* (91, 11%); on the other hand, the three species (*A. coluzzii*, *A. gambiae*, and *A. arabiensis*) were present in the village Tchabbal, with the proportion of *A. gambiae* much higher than that observed in the city of Ngaoundere. The strong dominance of *A. coluzzii* in the town of Ngaoundere could be explained by the ecological conditions that prevail there. It is a species that likes sites located near water bodies, prefer habitats with higher annual rainfall, higher water vapour pressure, lower temperatures and lower evapotranspiration [28]. Ngaoundere is an area irrigated by many rivers; and 1.500 mm rain fall average per year, with an average temperature of 22 °C. All these framework parameters with the ecological preferences of *A. coluzzii* mentioned above. *A. gambiae* for its part, has a preference for dry savannas, deciduous forest and warmer habitats [28]; which explains its higher proportion the village Tchabbal than the city of Ngaoundere. Concerning *A. arabiensis*, it generally exhibits the same ecological preference as *A. gambia*e [28]. In addition, we can also add its opportunistic throphic behavior. Although more anthropophilic, it gorges more readily cattle than *A. gambiae* and *A. colluzzii* [29]. As cattle rearing is much accentuated in the locality of Chabbal, this would better promote the development of *A. arabensis*, unlike the city of Ngaoundere where urbanization is very intensive.

Concerning the screening of the mechanisms involved in resistance to the tested insecticides, we observed a predominance of the 1014(F) mutation of the kdr gene. It is nowadays clearly established that this mutation of the kdr gene is associated with resistance of *A. gambiae s.l.* to pyrethroids [30]. However, the kdr gene is not solely responsible for mosquitoes resistance to pyrethroids; Pre-exposure of mosquitoes to PBO led to an increased in mortality rate to pyrethoid insecticides, thus signaling an involvement of P450 oxidases in this resistance [31]. The Reversion of resistance driven by the synergist PBO not only signals the involvement of P450 in this resistance [31], but also offers an interesting alternative for the management of resistance to pyrethroids. Experimental studies conducted by the Cochrane group in Kenya in recent years showed that mosquito nets impregnated with a pyrethroid+PBO were more effective in areas where there was a strong resistance of the kdr type [27]. New vector control approaches therefore envisage promoting a new generation of bi-impregneted mosquito nets with PBO + pyrethroid.

Conclusion

This study shows a good knowledge of malaria in the studied populations. Mosquito nets are the main tool used to prevent malaria. There is a strong presence of the 1410F allele responsible for resistance to pyrethroids in local anophelines, which could justify the decrease in the bioeffectiveness of LLINs on these vectors. Prior exposure of mosquitoes to PBO increased their mortality rates to pyrethoids. The use of mosquito nets impregnated with PBO + pyrethoids could improve vector control in these localities.

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