OPEN ACCESS

GAVIN PUBLISHERS

Current Trends in
Entomology and Zoological Studies

Mbida AM, et al. Curr Trends Entomol: CTET-106.

Research Article

DOI: 10.29011/CTEZS-106. 100006

Insecticidal Nets Versus kdr-Resistant Mosquitoes

Arthur Mbida Mbida[1], Patrick Akono Ntonga[1], Parfait Awono-Ambene[2], Romeo Tchoffo[1], Abdou Talipouo[1], Wolfgang Ekoko[1,2], Jérome Binyang[1,3], Alain Dongmo[1], Remy Mimpfoundi[3], Josiane Etang[2,4,5*]

[1]Laboratory of Animal Biology and Physiology, University of Douala, Douala, Cameroon

[2]Organization Coordination Organization for the Control of Endemic Diseases in Central Africa (OCEAC), Yaoundé, Cameroon

[3]Laboratory of General Biology, University of Yaoundé I, Yaoundé, Cameroon

[4]Department of biological Sciences, Faculty of Medicine and Pharmaceutical Sciences, University of Douala, Douala, Cameroon

[5]Institute for Insect Biotechnology, University of Giessen, Germany

[*]**Corresponding author:** Josiane Etang, Institute for Insect Biotechnology, University of Giessen, Germany, Winchesterstr. 2, 35394 Gießen, Germany. Tel: +496419939502; Fax: +49 6414808581; Email: josyet2@gmail.com

Citation: Mbida AM, Ntonga PA, Awono-Ambene P, Tchoffo R, Talipouo A, et al. (2018) Insecticidal Nets versus kdr-Resistant Mosquitoes. Curr Trends Entomol: CTET-106. DOI: 10.29011/CTEZS-106. 100006

Received Date: 21 February, 2018; **Accepted Date:** 21 March, 2018; **Published Date:** 30 March, 2018

Abstract

The current study aimed at assessing the coverage and bio efficacy of Long Lasting Insecticidal Nets (LLINs) against *Anopheles (An.) coluzzii*, the main malaria vector in Manoka and Youpwe (Littoral Cameroon), known as being resistant to pyrethroid insecticides.

A cross sectional LLINs survey was conducted in the Manoka island rural area and the Youpwe urban area of the Douala town between December 2014 and April 2015. The bio-efficacy of field collected LLINs against *An. gambiae.s.l*. from the Kisumu laboratory colony and two field samples was assessed using WHO cone test. Mosquito specimens (dead and survivors) following cone test were identified down to species using a PCR-RFLP method and genotyped for kdr L1014 resistant mutations using Hot Oligonucleotide Ligation Assay.

The household ownership of LLINs was 73-81% in Manoka and Youpwe (X^2=1.40; p=0.24). However, the universal coverage ratio (2 people/net) was not achieved. Less than 40% LLINs were physically damaged, with 9.9 (±1.8) holes/net. Generally, torn LLINs were still usable and effective against the Kisumu *An. gambiae* s.s. strain (100% knockdown and 83-96% mortality rates). However, LLNs were no longer effective against *An. gambiae s.l.* populations from Manoka and Youpwe (knockdown and mortality rates <15%), essentially composed of *An. coluzzii* species. The frequencies of kdr 1014F insecticide resistance allele in surviving *An. coluzzii* samples were 75-88%.

This study revealed good functionality of LLINs against susceptible *An. gambiae* s.s. However, the long lasting efficacy against mosquito bites and malaria transmission expected at community level may be hindered by *An. coluzzii* resistance to insecticides.

Keywords: *Anopheles coluzzii*; Bed nets; Cameroon; Insecticide resistance; Kdr 1014F mutation; Malaria

Abbreviations

An	:	*Anopheles*
DDT	:	Dichloro-diphenyl-trichlorethane;
DNA	:	Deoxyribonucleic acid
HOLA	:	Hot oligonucleotide ligation assay
Kdr	:	Knockdown resistance
LLINs	:	Long-lasting insecticidal nets
L1014F	:	Leucine to phenylalanine mutation at 1014 locus

L1014S : Leucine to serine mutation at 1014 locus

OCEAC : Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale

OMS : Organisation Mondiale de la Santé

PCR-RFLP : Polymerase chain reaction-restriction fragment length polymorphism

KDT_{50} : Time of knockdown for 50% tested specimens

WHO : World Health Organization

NMCP : National Malaria Control Programme

Introduction

Malaria remains one of the most deadly diseases in the world, devastating people's health and livelihoods. In 2016, the number of malaria cases worldwide was estimated at 216 million compared 212 million in 2015, leading to 445 000 deaths ≈ 446 000 in 2015, most of which in children aged under 5 years (92%) in Africa [1].Though, back to year 2000, the incidence has been reduced by 41% and mortality rates by 62%, there was a noticeable increase of malaria burden between 2015 and 2016. To sustain the progress towards malaria elimination, the "Global Technical Strategy for Malaria 2016-2030" (GTS) [2] and the Roll Back Malaria Advocacy Plan called "Action and investment to defeat malaria 2016-2030" (AIM)- for a malaria-free world [3] emphasize the need for universal access of vulnerable populations to interventions for prevention, diagnoses and treatment.

Current malaria mass prevention measures include vector control that aims at stopping mosquitoes from biting humans and mass drug administration that suppress infections where appropriate. Sleeping under an ITN or in a house treated through insecticide indoor residual spraying (IRS) are the common vector control measures. In few specific settings and circumstances, ITNs and IRS can be supplemented by larval source management [4] or other environmental measures or improvement of housing [5] that reduce the suitability of the environment as mosquito habitats or that otherwise restrict the human biting rates. ITNs have a dual action, i.e. a physical barrier preventing the human-mosquito contact and a chemical barrier provided by the insecticide which kills mosquitoes that come into contact with the net. The efficiency of ITNs therefore depends not only on their physical integrity, coverage and good practices of usage, but also on their killing effect on local anopheline species which provides the chemical barrier [6,7]. Monitoring these parameters at community level is therefore useful for the planning of ITN renewal.

Pyrethroids are the only class of insecticides recommended for net treatment [7]. They have the advantage of speedy action against mosquito populations and very low toxicity to humans. However, there is a growing concern that malaria vectors are becoming more and more resistant to the pyrethroid insecticides. Resistance to DDT and pyrethroid insecticides has rapidly evolved and is now wide spread in the main malaria vectors in Africa, including species of the *An. gambiae* complex and those of the *An. funestus* group [8-10]. This resistance is attributed to kdr L1014F or L1014S mutations, elevated activity of glutathione s-transferases, P450 oxidases and esterases [11-14]. For the time being, the impact of insecticide resistance on malaria burden as a public health problem has been harder to demonstrate, for reasons that remain unclear. One possible reason is suggested that although resistant mosquitoes are surviving contact with the insecticide, the development of malaria parasites inside those mosquitoes is affected by the chemicals [15]. However, the transmission dynamics model predicts that the higher the population prevalence of pyrethroid resistance the greater impact it will have on both the number of clinical cases and the force of infection (as measured by the entomological inoculation rate). This is due to the lower initial killing efficacy of the LLIN, but also because of the higher rate of decay of insecticidal activity (it gets less effective more quickly) [16,17]. In order to sustain the efficacy of ITNs, action should urgently be taken to manage pyrethroid resistance while waiting for new active ingredients to be commercialized. According to GPIRM [18], the choice of insecticide for malaria vector control should always be informed by recent local data on the susceptibility of target vectors to insecticides as well as data on the expected efficacy of the prequalified vector control tool.

In Cameroon, over 90% of population is at risk of malaria [19]. Malaria infection is essentially due to *Plasmodium falciparum*, with few *P. malariae.* The transmission of these parasites is exacerbated by anthropic environmental changes and climatic conditions leading to proliferation of anopheline vector's habitats [20]. Several *Anopheles* species have been incriminated as vectors, mainly *An. arabiensis*, *An. coluzzii* and *An. gambiae*, three species of the *Anopheles gambiae* complex, those of the *An. funestus* group, *An. moucheti* and *An. nili* [21]. The geographical distribution of these species varies among eco-epidemiological facies. In the southern equatorial areas, malaria prevalence is high; the transmission is perennial and broadly ensured by *An. gambiae* and *An. coluzzii*. More interestingly, *An. coluzzii* is the main vector species breeding in salt water coastal areas of the Littoral Region [20,22]. This vector species is highly receptive of insecticide resistance alleles such as the kdr 1014F, which is widespread in *An. gambiaes.l.* populations from Cameroon [23], as well as in many other countries in Africa [11]. The rapid evolvement of insecticide resistance in malaria vectors from Cameroon is seen as a big obstacle towards malaria elimination in this country. ITNs are the primary vector control tools in Cameroon [24]. Eight million branded PermaNet® long-lasting insecticidal nets (deltamethrin coated LLINs) have been distributed by the National Malaria

Control Programme (NMCP) in the first nationwide campaign organized in 2011. The second nationwide LLIN free distribution campaign was organized in 2015-2016. The achievements of these LLINs campaigns in reducing malaria burden as well as the appropriate time for LLIN replacement need more attention.

The current study was conducted in the Manoka island rural area and the Youpwe urban area in the Littoral Region of Cameroon, where *An. coluzzii,* the major malaria vector species, was recently reported to develop resistance to pyrethroids, with high frequency of kdr 1014F allele [23]. The aim was to assess the ownership, usage and functionality of LLINs, as well as their residual bio-efficacy against the local *An. coluzzii* population, 29 months after the 2011free mass distribution campaign, in order to guide the decision of net renewal in 2015.

Materials and Methods

Study Sites

The study was carried out from December 2014 to April 2015 in two wetlands of the Wouri river estuary in Douala, the capital city of the Littoral Region in Cameroon: the Manoka rural island area (03°47' N; 09°39' E) and the Youpwe urban inland area (04°00' N; 09°42' E). The Littoral region is characterized by a sub equatorial climate with two seasons, a dry season from November to March and a rainy season from April to October. The average rainfall ranges from 3,600 to 10,000 mm of rain per year [24], with 80% relative humidity and average temperatures between 27°C to 29°C. Its driest month is December (28 mm average rain fall), while its wettest month is August (700 mm average rain fall) [24]. The human population is cosmopolitan, including natives (Bakoko and Malimba) and people from abroad such as Nigerians, Malians and Ghanaians. ITNs are the main control tools in households, complemented by coils, mates, repellents and sprays in some houses. In the both localities, *An. coluzzii* is the main malaria vector species [20,22].

Manoka is a rural area of approximately 365 km^2, with 602 inhabitants and around 100 households grouped in ten camps (Nyangadou, Bord, Dahomey, Plateau, Buea I, Epaka I & II, Number One Creek, Number Two Creek and Sandje). The main activities of the local communities are fishing and trade, small livestock, hunting and subsistence agriculture. Human dwellings are built on stilts. Because the soil is sandy and does not retain enough water, mosquito natural breeding sites are few compared with semi-permanent artificial breeding sites.

Youpwe is an urban area of about 1.3 km^2. The general population was estimated at 3,200 inhabitants grouped in800 households in 2005. The main activities of the local population are fishing and trade. Increasing exploitation of sand and wood led by the increase of population and unplanned urbanization has caused significant changes in the environment and proliferation of mosquito breeding sites.

Household Survey for LLINs Coverage, Utilization and Integrity

This was a cross-sectional and community-based survey conducted from December 2014 to April 2015 in Manoka and Youpwe neighborhoods. Authorization to conduct the survey was first sought from Chiefs or Quarter heads and each community was only investigated with their approval. The population was then adequately sensitized on the project objectives, methods and possible benefits/risks.

In the Manoka island, all households belonging to four selected camps (Nyangadou, Dahomey, Manoka, and Plateau) were visited, making a total of 100. Hundred out of 800 households living in Youpwe were selected following a step of 5 households along the way and visited. Prior to participant enrollment, clear explanation was given on the aim of the study, and written informed consent was obtained. Participants were only enrolled if they or their caregivers/guardians gave written informed consent. A household could include a single individual of either gender, or a compound with several people. In each household, one or both parents present were interviewed to obtain the information needed in either English/French (national languages) or pidgin (local language). Households were interviewed about the source, number and utilization of nets when available (e g, do the net have any holes and, if so, how many). Household heads were also asked for the number, age and gender of family members, and the population at-risk (children less than five years old and pregnant women) was recorded.

Additional information on the brand, hanging, physical integrity and utilization of nets were obtained through direct observation. A net was considered in utilization when it was hanged, and its owner declared having used it during the last three nights before the survey. All sides of installed nets were checked to detect holes or tears. Six indicators of LLINs coverage were evaluated:

- Ownership defined as the proportion of households owning at least one LLIN
- Universal coverage (households with at least one LLIN for every two people)
- Operational coverage (at-risk population under universal coverage of LLINs) [25]
- Usage (proportion of the population that used LLINs the three-previous night)
- Effective coverage (coverage of LLINs in functionally good conditions)

- LLIN physical integrity defined as the sizes of holes [26].

Long Lasting Insecticidal Net Sampling and Storage

Ten LLINs were collected from ten surveyed households selected in Manoka (5 households) and Youpwe (5 households). At least 100m 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, five 25 cm^2 pieces of netting were removed (one piece per side of the net) according to the WHO protocol [27], wrapped in aluminum foil, labeled and stored at 4˚C for subsequent bio assays with field and laboratory mosquito samples.

Mosquito Collection and Rearing

Immature Anophelines were collected from breeding sites in Manoka in December 2014 (i.e. during the dry season) and Youpwe in April 2015 (i.e. during the rainy season). In each study site, a 1.5-2 km^2 area was inspected and all water bodies checked [22]. Mosquito larvae and pupae were collected by dipping method and reared until adult stage in local insectariums. Emerging adult mosquitoes were morphologically identified using reference keys [28,29] and those belonging to the *An. gambiae* complex were kept for the bioassays. Specimens of the Kisumu reference *An. gambiae* s.s laboratory colony, originated from Kenya and maintained since several years in OCEAC (Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale) were brought to the study sites and reared in the local conditions, for used as control.

Laboratory Testing and Analysis

Cone Bioassay

The residual bio-efficacy of ITNs was assessed using the WHO cone test [30]. 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 thirty 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 Samples

Total DNA of dead and survivor mosquitoes after bioassays, was extracted following the protocol described by Collins et al. [31]. Molecular identification was performed with each extract using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) as described by Fanello et al. [33]. Alleles at locus 1014 were identified using Hot Oligonucleotide Ligation Assay (HOLA) [34].

Data Analysis

All the collected data were entered in Microsoft Excel 2013 sheets and graphs were drawn. Statistical analysis was performed using the statistical package for social sciences (SPSS) version 20.0 software.

The coverage rate was determined by dividing the total number of nets available in households by the total number of nets expected (half the total number of inhabitants). Protection of sleeping places (beds, mats, mattresses and floor) was determined by dividing the total number of nets available by the total number of sleeping places to be protected in households. Nets utilization rate was obtained by dividing the total number of nets in use by the number of nets received and available in the community. Tears and holes on nets were classified into four categories according to their size [26]:

- size 1 (S_1) : 0.5-2cm,
- size 2 (S_2) : 2-10cm,
- size 3 (S_3) : 10-25cm,
- size 4 (S_4) : more than 25cm.

These categories were used to calculate the proportionate holes-index (pHI), which is the indicators of wear (WHO 2011), as follows:

$$pHI = (a \times \text{number of } S_1 \text{ holes}) + (b \times \text{number of } S_2 \text{ holes}) + (c \times \text{number of } S_3 \text{ holes}) + (d \times \text{number of } S_4 \text{ holes});$$

a, b, c and d being the weighting factors (a=1, b=23, c=196 and d=578),which correspond to the areas of estimated holes on the assumption that the hole sizes in each category are equal to the mid-points.

Based on pHI, nets were classified into three categories:

- good nets (0<pHI <64), , i.e. there is no reduction of physical barrier compared with undamaged nets;
- acceptable nets (65<pHI <642), i.e. the physical barrier is somewhat reduced but still provide significantly more protection than no net at all;
- Disposable nets (pHI >642), i.e. the protection for the user is in serious doubt and the nets should be replaced as soon as possible.
- A Chi-square test was used for comparison of LLIN ownership, coverage and usage. A post hoc analysis was performed using a Dunnet test to make pair wise comparisons of mean hole-

Citation: Mbida AM, Ntonga PA, Awono-Ambene P, Tchoffo R, Talipouo A, et al. (2018) Insecticidal Nets versus kdr-Resistant Mosquitoes. Curr Trends Entomol: CTET-106. DOI: 10.29011/CTEZS-106. 100006

indexes between Manoka and Youpwe. The level of significance was set at P values<0.05.

Knockdown and mortality rates of mosquitoes' post exposure to LLINs were calculated and analyzed according to World Health Organization's criteria [30]. A net was considered effective when mortality rate in exposed mosquitoes was > 80% and Knockdown rates > 95%.

Results

Long Lasting Insecticidal Netowner ship, Coverage and Utilization

Data from the LLIN survey in Manoka and Youpwe are summarized in Table 1. A total of 200 households were visited, including 100 households sheltering 662 people in Manoka and 100 households sheltering 605 people in Youpwe. All the LLINs recorded (404 LLINs) in the two communities were of Permanet 2.0 brand. LLIN ownership in households was not significantly different between Manoka (73%) and Youpwe (81%) (X^2=1.40; p=0.24).

Indicators	Manoka	Youpwe
Number of households surveyed	100	100
Human population in surveyed households	662	605
Number of at risk persons (children< 5 years and pregnant women)	116	130
Number of sleeping spaces	419	312
Number of nets recorded	235	169
Mean number of nets per household	2.4 ± 0.50	1.7 ± 0.13
Mean number of people per net	2.8 ± 0.7	3.5 ± 1.16
Number of households owning at least one net (%)	73 (73.00)	81 (81.00)
Number of households owning at least one net for 2 peoples (%)	38 (38.00)	26 (26.00)
Number of children< 5 years and pregnant women covered (%)	59 (50.86)	101 (77.69)
Number of people covered (%)	471 (71.14)	338 (55.86)
Number of sleeping spaces covered (%)	235 (56.08) 221 (94.04)	169 (54.16)
Number of nets regularly used (%)		158 (93.49)

Table 1: Long Lasting Insecticidal nets' coverage and domestic utilization.

LLIN's coverage (number of people covered bed nets) was higher in Manoka (71%) than Youpwe (56%) (X^2=34.27; p<0.001). Conversely, the operational coverage (proportion of the at-risk population covered) was lower in Manoka (51%) and compared with Youpwe (78%) (X^2=19.56; p<0.001). In both settings, the universal coverage (corresponding to at least one net for 2 persons) was not achieved in the general population, the coverage ratio was around one net for 2.40±0.70 and 3.5±1.16 people in Manoka and Youpwe respectively. Out of the households visited, 26% and 38% households achieved universal coverage of LLINs in Youpwe and Manoka respectively (X^2=1.00; p=0.32).

Among the 404 LLINs recorded in households, 379 nets were installed and regularly used. The rate of net utilization was around 94%. In both communities, 54-56% sleeping spaces were covered with LLINs. The 25 remaining nets were either kept in suitcases or used for any other purpose and not for malaria prevention.

Functionality of Long Lasting Insecticidal nets in use

Physical integrity

A total of 204 LLINs were assessed for their physical integrity, including 108 nets from Manoka and 96 nets from Youpwe. The breakdowns of mean hole-sizes encountered per net and the distribution of good, acceptable or disposable nets according to proportionate hole indexes are provided in Tables 2 and 3 respectively.

Locality	Mean number of holes of each size per net				
	Size I	Size II	Size III	Size IV	(p-value)
Manoka	3.82 ± 0.22	2.53 ± 0.31	1.12 ± 0.16	0.15 ± 0.09	p<0.001
Youpwe	6.08 ± 0.57	4.70 ± 0.66	1.36 ± 0.24	0.36 ± 0.11	p<0.001
Overall	4.9 ± 0.39	3.6 ± 0.48	1.24 ± 0.20	0.2 ± 0.10	p<0.001

Table 2: Hole indexes of Long Lasting Insecticidal Nets.

	Manoka	Youpwe
Number of nets assessed	108	96
Number of nets with holes (%)	34 (31.50)	36 (37.50)
Number of good nets (0<pHI<64) (%)	10 (28.70)	8 (23.96)
Number of acceptable nets (65<pHI<642) (%)	22 (65.71)	14 (40.62)
Number of disposable nets (pHI>642) (%)	2 (5.72)	12 (35.42)
Mean pHI	368.23	588.82
pHI: proportionate hole insdex		

Table 3: Categorization of Long Lasting Insecticidal Nets under domestic utilization.

Less than 40% of examined nets were torn, with 34-36% LLINs showing at least one hole. A total of 658 holes were recorded on 70 LLINs, the average number of holes per net was 9.9 (±1.8). The average number of holes significantly decreased from S1 (3.82 ± 0.22 holes/net) to S4 (0.15 ± 0.09 holes/net) in Manoka (P<0.001), or from S1 (6.08 ± 0.57 holes/net) to S4 (0.36 ± 0.11holes/net) Youpwe (P<0.001). Indeed, S1 and S2 holes were mostly found on nets compared with S3 and S4 holes.

Based on the proportionate hole-index, 29% and 24% LLINs from Manoka and Youpwe respectively were found good (X^2=0.08, p=0.77). In Manoka, acceptable nets were predominant (≈66%), while only few nets (≈ 6%) were disposable ($9.70 \leq X^2 \leq 27.71$; p<0.001). In Youpwe, acceptable nets (41.2%), good nets (23.5%) or unusable nets (35.2%) were equally represented ($0.26 \leq X^2 \leq 2.56$, $0.10 \leq p \leq 0.60$). Overall, the torn LLINs were acceptable with 368-589 pHI, which is less than the WHO threshold for disposable nets (pHI=642).

Residual bio efficacy

A total of 2,038 mosquito specimens of the Kisumu susceptible reference *An. gambiae* s.s. strain were exposed to 50 pieces of nettings, including 25 nettings (5 pieces * 5 LLINs) from Manoka and 25 nettings (5 pieces * 5 LLINs) from Youpwe. One thousand six (1,006) *An. gambiae s.l.* specimens from Manoka were exposed nettings from Manoka, while 1,004 *An. gambiae s.l.* specimens from Youpwe were exposed to netting from Youpwe. For each batch of 25 tested nettings, 100 mosquito specimens were exposed to untreated nets as negative control. Knockdown and mortality rates of the three mosquito strains after exposure to LLINs are summarized in (Figure 1).

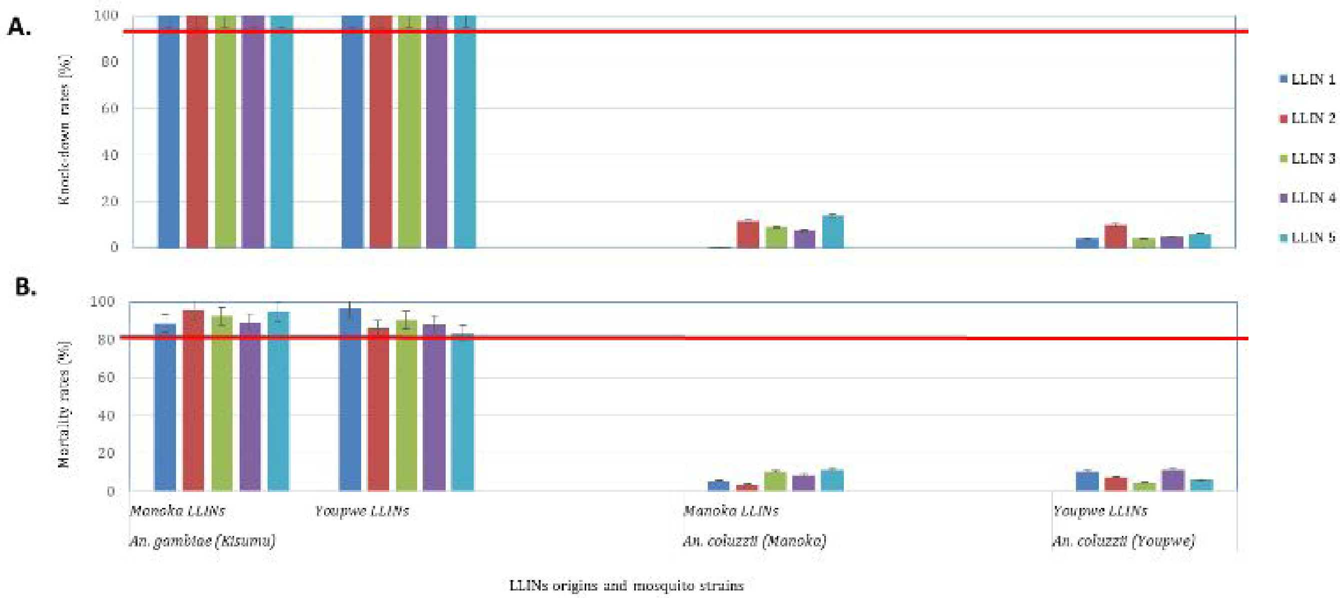

Figure 1: Knock-down and mortality rates of susceptible *An. gambiae* s.s and *An. coluzzii* strains to Long Lasting Insecticidal Nets (LLINs) from Manoka and Youpwe.

A. Knock-down rates 60minutes post 3minutes exposure to Long Lasting Insecticidal Nets

B. Mortality rates 24h post exposure to Long Lasting Insecticidal Nets

Red lines: Knockdown and mortality threshold of LLINs bio-efficacy [26].

The knockdown and mortality rates in negative control samples were less than 5%. All the ten tested LLINs were found effective against the Kisumu strain. The knock-down rates were 100% and the mortality rates 83-96%. However, LLINs were no longer effective against field collected *An. gambiaes.l.*, either from Manoka or from Youpwe. The Knockdown and the mortality rates were repeatedly below 15%.

Mosquito species and kdr L1014 genotypes

Overall,180 anopheline specimens morphologically identified as belonging to the *An. gambiae* complex and previously used for testing LLINs residual efficacy were identified down to species. These samples were randomly selected among dead (N=30) and survivor (N=60) mosquitoes from each study site (90 from Manoka and 90 from Youpwe).

All the analyzed samples were essentially made up with *An. coluzzii* species. No *An. melas* or *An. gambiae* s.s. species was identified although the two study sites are located in the coastal areas. In survivor *An. coluzzii* samples, two kdr alleles (susceptible 1014L and resistant 1014F) were detected (Table 4). The frequency of the 1014F allele was high and not significantly different between the *An. coluzzii* samples from Manoka (75 %) and Youpwe (88 %) (X^2=3.36; p=0.07). In dead *An. coluzzii* samples, only the kdr susceptible 1014L allele was found (100% frequency).

			Allele frequencies in kdr locus (%)[CI_{95}]		
Location		N	1014L (S)	1014F (R_w)	1014S (R_e)
Manoka	Survivors	60	25 [16.4-35.4]	75 [64.6-82.6]	0
	Dead	30	100	0	0
Youpwe	Survivors	60	12 [7.1-17.6]	88 [82.3-93.1]	0

	Dead	30	100	0	0
N=Sample size, CI95=confidence interval at 95%, 1014L=1014 Leucine, 1014F=1014 Phenylalanine, 1014S=1014 Serine					

Table 4: Kdr 1014 allelic frequencies in *An. coluzzii* samples from Manoka and Youpwe.

Discussion

The cross-sectional survey conducted at community level revealed a noticeable LLIN ownership at household level (73-81%) and regular utilization of available LLINs (93-94%). LLINs ownership recorded in both study sites was similar to that reported in the South West Region of Cameroon (72-83%) [34]. Apart from the nationwide free LLIN distribution campaign undertaken in 2011, LLINs could also be procured from antenatal services or from the private sector.

Different patterns of LLINs coverage of population at risk (children under 5 years' old and pregnant women) and general population were observed in Manoka (51% and 71% respectively) and Youpwe (78% and 56% respectively). The low coverage of the at-risk population in Manoka may be associated with the low assess of this rural community to antenatal LLINs distribution services contrarily to the Youpwe urban area where health facilities are close to the community. Most of the LLINs in Manoka may therefore come from the nationwide distribution campaign, while several LLINs distribution channels may exist in Youpwe. Indeed, the proportion of general population covered was higher in Manoka compared with Youpwe where alternative measures against mosquito bites and better living conditions (fans, air conditioner, coils, mats…) likely led to decreased interest to LLIN.

The discrepancy of LLINs coverage in the two surveyed communities was illustrated by the number of LLINs available in each community versus the human populations surveyed. In general, a significant gap to achieve universal coverage of LLINs (one net for 2 people) was clearly revealed in both settings. These data are consistent with those reported by Kodila-Tedika [35] as well as the Ministry of Public health in Cameroon in 2013; i.e. 2 years after the first nationwide free distribution campaign of LLINs [24], prompting the implementation of the second nationwide LLIN distribution campaign in 2015.

However, most of the nets available in households were serviceable; the general proportion of torn LLINs was less than 40%. The holes encountered on LLINs were mainly of S_1 and S_2 sizes, while S_3 and S_4 holes were scares. The proportion of torn LLINs recorded in this study is two times lower than 89.39% previously reported in South West Cameroon [34]. In Uganda, considerable physical damage (45-78% of damaged nets) was reported even within a year of bed net domestic use [36]. The good retention of LLINs in Manoka and Youpwe may result from community awareness of LLIN utility in the protection against mosquito bites and malaria. Nevertheless, S_1 and S_2 holes may gradually develop into S_3 and S_4, and render the net disposable. It is therefore important to educate the communities about how and when to repair torn bed nets.

It is noteworthy that ITNs are expected to provide a physical barrier to mosquito bites as well as repellent, knockdown and killing effects from insecticide treatment. A recent meta-analysis found that ITNs were significantly protective than untreated nets even in the presence of pyrethroid resistance in local vectors [38]. Furthermore, LLINs were reported to offer individual protection against malaria infection in an area of high resistance [38]. On top of personal protection, if the coverage of nets is high enough there is a mass protection [39] which benefits both users and non-users by reducing the lifespan of mosquitoes. Although universal LLINs coverage was not achieved in most of the surveyed households in Manoka and Youpwe, the overall coverage of the general population e.g. 71% in Manoka could lead to a certain level of mass protection of the target communities. The mass protection was further expected from the time when field collected LLINS were found physically acceptable and highly effective against the Kisumu susceptible strain of *An. gambiae,* despite their age (29 months) and regular utilization in domestic conditions.

However, the drastic decrease of residual bio-efficacy of LLINs against field mosquito samples revealed that resistance may attenuate their mass protection effect. In this context, the personal protection would arise only from physical integrity of nets, which may not be optimal as expected by the Malaria Vector Control Programme.

Molecular identification of target malaria vector species and screening of resistance mechanisms are informative methods for the monitoring of the bio efficacy of insecticide-based vector control tools [40].In this study, *An. coluzzii* was the only malaria vector identified in field collected anopheline samples. The predominance of this species in the estuary of the Wouri River is consistent with previous studies [23,41]. The high frequency of kdr 1014F mutations recorded in the identified *An. coluzzii* samples stresses that "knockdown resistance" kdr 1014F mutation is one of the major resistance mechanisms to DDT, permethrin and deltamethrin in surveyed settings as previously reported [23]. While the kdr 1014S allele was not recorded in analyzed samples, this allele was already reported in Douala although at very low frequency [14]. *An. coluzzii* is highly receptive of kdr 1014 knockdown resistance alleles, which are currently spreading in species of the *An. gambiaes.l.* complexin Cameroon [14], as well as in many other countries in Africa [12]. The current data therefore call attention monitoring LLINs bio efficacy against local malaria vector populations and timely replacement of nets, as well as implementation of alternative vector control tools based on eco-epidemiological features. For the communities living across the Wouri estuary, other tools are need in addition to LLINs, in order to strengthen malaria vector control. The main economic activity in these areas is fishing; which is organized to night. This activity may therefore lead to the decrease of the number of people sleeping under mosquito. Since permanent and manmade mosquito breeding sites have been mapped in both study sites [22] larval source management may be use as a complementary vector control measure in Youpwe and Manoka.

Conclusion

The current study revealed a worthy LLIN retention in Manoka and Youpwe, though universal coverage was not achieved. However, LLINs were no longer effective against the local *An. coluzzii* populations carrying knockdown resistance 1014F allele, threatening the mass protection against mosquito bites. These data are useful for LLIN replacement and for filling the gap towards universal coverage. More attention should also be given to monitoring LLIN residual efficacy against local malaria vector populations, in order to better plan LLINs replacement. Complementary strategies such as larval source management should also be promoted for insecticide resistance management and increasing the protection of people out of bed nets.

Acknowledgements

This study results from the collaboration between the University of Douala and Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale (OCEAC). We thank the population of Manoka and Youpwe for their kind collaboration.

References

1. World Health Organization (2017) World malaria report 2017: 196.

2. World Health Organization (2015) Global technical strategy for malaria 2016-2030: 29.

3. World Health Organization on behalf of the Roll Back Malaria Partnership Secretariat (2015) Action and investment to defeat malaria 2016-2030": 99.

4. World Health Organization (2013) Larval source management: A supplementary measure for malaria vector control (operational manual): 116.

5. Reck JC, Alegana V, Arinaitwe E, Cameron E, Kamya MR (2018) Rapid improvements to rural Ugandan housing and their association with malaria from intense to reduced transmission: a cohort study. Lancet Planet Health 2: 83-94.

6. World Health Organization (2007) Technical consultation on specifications and quality control of netting material for mosquito nets: 28.

7. Zaim M, Aitio A and Nakashima N (2000) Safety of pyrethroid-treated mosquito nets. Med Vet Entomol 14: 1-5.

8. Hemingway J, Lindsay SW, Small GJ, Jawara M, Collins FH, et al. (1995) Insecticide susceptibility status in individuals species of *Anopheles gambiae* complex (Diptera: Culicidea) in an area of the Gambia where pyrethroid impregnated bed nets are used extensively for malaria control. Bull Entomol Res 85: 229-234.

9. Hargreaves K, Koekemoer LL, Brooke BD, Hunt RH, Mthembu J, et al. (2000) *Anopheles funestus* resistant to pyrethroid insecticides in South Africa. Med vet entomol 14: 181-189.

10. Etang J, Manga L, Chandre F, Guillet P, Fondjo E et al. (2003) Insecticide susceptibility status of *Anopheles gambiae s.l.* (Diptera: Culicidae) in the Republic of Cameroon. J Med Entomol 40: 491-497.

11. Etang J, Fondjo E, Chandre F, Morlais I, Brengues C et al. (2006) First report of the kdr mutations in the malaria vector *Anopheles gambiae* from Cameroon. Am J Trop Med Hyg 74: 795-797.

12. Santolamazza F, Calzetta M, Etang J, Barrese E, Dia I, et al. (2008) Distribution of knockdown resistance mutations in *Anopheles gambiae* (Diptera:Culicidae) molecular forms in West and Central-West Africa. Mal J 7: 74.

13. Etang J, Vicente JL, Nwane P, Chouaibou M, Morlais I, et al. (2009) Polymorphism of intron-1 in the voltage-gated sodium channel of *Anopheles gambiae* s.s. populations from Cameroon with emphasis on insecticide knockdown resistance mutations. Mol Ecol 18: 3076-86.

14. Nwane P, Etang J, Chouaïbou M, Toto JC, Koffi A et al. (2013) Multiple insecticide resistance mechanisms in *Anopheles gambiae* s.l. populations from Cameroon, Central Africa. Parasit & Vectors 6: 41.

15. Kristan M, Lines J, Nuwa A, Ntege C, Meek SR, et al. (2016) Exposure to deltamethrin affects development of *Plasmodium falciparum* inside wild pyrethroid resistant *Anopheles gambiae* s.s. mosquitoes in Uganda. Parasites & Vectors 9: 100.

16. Churcher TS, Lissenden N, Griffin JT, Worrall E, Ranson H (2016) The impact of pyrethroid resistance on the efficacy and effectiveness of bednets for malaria control in Africa. Elife 5: e16090.

17. Etang J, Pennetier C, Piameu M, Bouraima A, Chandre F et.al. (2016) When intensity of deltamethrin resistance in *Anopheles gambiae* s.l. leads to loss of Long Lasting Insecticidal Nets bio-efficacy: a case study in north Cameroon. Parasites & Vectors 9: 132

18. World Health Organization (2012) Global Plan for Insecticide Resistance Management in Malaria Vectors (GPIRM). In: WHO/HTM/GMP/20125. Edited by Organization WH. Geneva, Switzerland: World Health Organization: 130.

19. MINSANTE (Ministère de la santé publique) (2006) Plan stratégique national de lutte contre le paludisme au Cameroun 2007-2010: 129.

20. Mbida AM, Etang J, Akono Ntonga P, Talipouo A, Awono-Ambene P, et al. (2016) Preliminary investigation on aggressive culicidae fauna and malaria transmission in two wetlands of the Wouri river estuary, Littoral-Cameroon. J Entomol Zool Stud 4: 105-110.

21. Antonio-Nkondjo C, HinzoumbeKerah C, Simard F, Awono-Ambene P, Chouaibou M, et al. (2006) Complexity of the malaria vectorial system in Cameroon: contribution of secondary vectors to malaria transmission. J Med Entomol 43: 1215-1221.

22. Mbida AM, Etang J, Akono Ntonga P, Eboumbou Moukoko C, Awono-Ambene P, et al. (2016) New Insight into *Anopheles coluzzii* Coetzee & Wilkerson, 2013 larval ecology in the Wouri estuary, Littoral-Cameroon. Bull Soc Pathol Exot 110: 92-101.

23. Etang J, Mbida AM, Ntonga Akono P, Binyang J, Eboumbou Moukoko CE, et al. (2016) *Anopheles coluzzii* larval habitat and insecticide resistance in the island area of Manoka, Cameroon. BMC Inf Dis 16: 217.

24. MINSANTE (Ministère de la santé publique) (2013) Enquête post campagne sur l'utilisation des moustiquaires imprégnées a longue durée d'action : 109.

25. World Health Organization (2013) Methods for achieving universal coverage with long-lasting insecticidal nets in malaria control. Report to MPAC September 2013.

26. World Health Organization (2011) Guidelines for monitoring the durability of long-lasting insecticidal mosquito nets under operational conditions: 44.

27. World Health Organization (2012) Report of the fifteenth WHOPES working group meeting. Geneva: World Health Organization.

28. Gillies MT, Coetzee MA (1987) Supplement to the Anophelinae of Africa south of the Sahara (Afrotropical region). Johannesburg: The South African Institute of Medical Research: 143.

29. Gillies MT, De Meillon B (1968) The Anophelinae of Africa South of the Sahara (Ethiopian zoogeographical region). In: 2nd ed. Johannesburg: The South African Institute of Medical Research: 343.

30. World Health Organization (2013) Guidelines for laboratory and field testing of long-lasting insecticidal nets. Geneva, World Health Organization: 1-89.

31. Collins FH, Mendez MA, Razmussen MO, Mehaffey PC, Besansky NJ, et al. (1987) Ribosomal RNA gene probe differentiates member species of *Anopheles gambiae* complex. Am J Trop Med Hyg 37: 37-41.

32. Fanello C, Santolamazza F, Della TA (2002) A Simultaneous identification of species and molecular forms of the *Anopheles gambiae* complex by PCR-RFLP. Med Vet Entomol 16: 461-465.

33. Lynd A, Ranson H, Mc Call PJ, Randle NP, Black IV WC, et al. (2005) A simplified high-throughput method for pyrethroid knockdown resistance (kdr) detection in *Anopheles gambiae*. Mal J 4: 16.

34. Boussougou-Sambe ST, Awono-Ambene P, Tasse Geraud CT, Etang J, Binyang JA (2017) Physical integrity and residual bio-efficacy of used LLINs in three cities of the South-west region of Cameroon 4 years after the first national mass-distribution campaign. Malar J 16: 31.

35. Kodila-Tedika O (2014) Education, paludisme et moustiquaires imprégnées d'insecticide en Afrique sub-saharienne: 26.

36. Kilian A, Byamukama W, Pigeon O, Atieli F, Duchon S, Phan C (2008) Long-term field performance of a polyester-based long-lasting insecticidal mosquito net in rural Uganda. Malar J 7: 49.

37. Strode C, Donegan S, Garner P, Enayati AA, Hemingway J (2014) The impact of pyrethroid resistance on the efficacy of insecticide-treated bed nets against African anopheline mosquitoes: systematic review and meta- analysis. PLoS Med 11: e1001619.

38. Bradley J, Ogouyèmi-Hounto A, Cornélie S, Fassinou J, Sissinto Savi de Tove Y, et al. (2017) Insecticide-treated nets provide protection against malaria to children in an area of insecticide resistance in Southern Benin. Malar J 16: 225

39. Hawley WA, Phillips-Howard PA, Kuile FOT, Terlouw DJ, Vulule JM, et al. (2003) Community-wide effects of permethrin-treated bed nets on child mortality and malaria morbidity in Western Kenya. Am J Trop Med Hyg 68: 121-7.

40. Donnelly MJ, Isaacs A, Weetman D (2016) Identification, validation, and application of molecular diagnostics for insecticide resistance in malaria vectors. Trends Parasitol 32: 197-206.

41. Wondji C, Simard F, Petrarca V, Etang J, Santolamazza F, et al. (2005) Species and populations of the *Anopheles gambiae* complex in Cameroon with special emphasis on chromosomal and molecular forms of *Anopheles gambiae* s.l. J Med Entomol 42: 998-1005.