Insecticidal Nets Versus kdr-Resistant Mosquitoes

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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.s. 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 (X²=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-trichloroethane;
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 Épidémies en Afrique Centrale
OMS : Organisation Mondiale de la Santé
PCR-RFLP : Polymerase chain reaction-restriction fragment length polymorphism
KDT50 : 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 to 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 in 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. gambiae s.l. populations from Cameroon [23], as well as in many other countries in Africa [11]. The rapid evolution 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 2011 free 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 km², 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 community 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 km². The general population was estimated at 3,200 inhabitants grouped in 800 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 nights)
- 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 cm² 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 km² 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 Endémiies 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 (S₁) : 0.5-2cm,
- size 2 (S₂) : 2-10cm,
- size 3 (S₃) : 10-25cm,
- size 4 (S₄) : more than 25cm.

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

\[
\text{pHI} = (a \times \text{number of } S₁ \text{ holes}) + (b \times \text{number of } S₂ \text{ holes}) + (c \times \text{number of } S₃ \text{ holes}) + (d \times \text{number of } S₄ \text{ holes});
\]

\[a, b, c \text{ and } d \text{ being the weighting factors (} a=1, b=23, c=196 \text{ and } d=578\text{),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-
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 Net ownership, 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).

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

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.
<table>
<thead>
<tr>
<th>Locality</th>
<th>Size I</th>
<th>Size II</th>
<th>Size III</th>
<th>Size IV</th>
<th>(p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manoka</td>
<td>3.82 ± 0.22</td>
<td>2.53 ± 0.31</td>
<td>1.12 ± 0.16</td>
<td>0.15 ± 0.09</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Youpwe</td>
<td>6.08 ± 0.57</td>
<td>4.70 ± 0.66</td>
<td>1.36 ± 0.24</td>
<td>0.36 ± 0.11</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Overall</td>
<td>4.9 ± 0.39</td>
<td>3.6 ± 0.48</td>
<td>1.24 ± 0.20</td>
<td>0.2 ± 0.10</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

Table 2: Hole indexes of Long Lasting Insecticidal Nets.

<table>
<thead>
<tr>
<th></th>
<th>Manoka</th>
<th>Youpwe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of nets assessed</td>
<td>108</td>
<td>96</td>
</tr>
<tr>
<td>Number of nets with holes (%)</td>
<td>34 (31.50)</td>
<td>36 (37.50)</td>
</tr>
<tr>
<td>Number of good nets (0&lt;pHI&lt;64) (%)</td>
<td>10 (28.70)</td>
<td>8 (23.96)</td>
</tr>
<tr>
<td>Number of acceptable nets (65&lt;pHI&lt;642) (%)</td>
<td>22 (65.71)</td>
<td>14 (40.62)</td>
</tr>
<tr>
<td>Number of disposable nets (pHI&gt;642) (%)</td>
<td>2 (5.72)</td>
<td>12 (35.42)</td>
</tr>
<tr>
<td>Mean pHI</td>
<td>368.23</td>
<td>588.82</td>
</tr>
</tbody>
</table>

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 ± 0.11 holes/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 (X²=0.08, p=0.77). In Manoka, acceptable nets were predominant (≈66%), while only few nets (= 6%) were disposable (9.70≤X²≤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≤X²≤2.56, 0.10≤p≤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. gambiae.s.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 %) (X²=3.36; p=0.07). In dead An. coluzzii samples, only the kdr susceptible 1014L allele was found (100% frequency).

<table>
<thead>
<tr>
<th>Location</th>
<th>Allele frequencies in kdr locus (%) [CI95]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1014L (S)</td>
</tr>
<tr>
<td>Manoka</td>
<td>Survivors</td>
</tr>
<tr>
<td></td>
<td>Dead</td>
</tr>
<tr>
<td>Youpwe</td>
<td>Survivors</td>
</tr>
</tbody>
</table>

Table 4: Allele frequencies in kdr locus (%) [CI95]
community awareness of LLIN utility in the protection against mosquito bites and malaria. Nevertheless, S1 and S2 holes may gradually develop into S3 and S4, 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

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<td>N=Sample size, CI95=confidence interval at 95%, 1014L=1014 Leucine, 1014F=1014 Phenylalanine, 1014S=1014 Serine</td>
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Table 4: Kdr 1014 allelic frequencies in An. coluzzii samples from Manoka and Youpwe.
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 mannmade 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.

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**References**
