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Comparative efficacy of five types of long-lasting insecticide-treated nets (PermaNet 3.0[®], PermaNet 2.0[®], Olyset Plus[®], Olyset Net[®], and LifeNet[®]) in a semi-natural environment against resistant *Anopheles gambiae sensu lato* and *Mansonia africana* in Cove, Benin

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Abstract

To increase the effectiveness of long-lasting insecticide-treated nets (LLINs) to reduce man-vector contact and to develop a rational management strategy of insecticide resistance, a new generation of LLINs treated with pyrethroids and piperonyl butoxide (PBO) was developed. The present study aimed to verify the effects of the LLINs against pyrethroid-resistant *Anopheles gambiae s.l.* populations in Benin.

The assessment of these LLINs was conducted at an experimental station located in a large rice-growing area in Cove district, Benin. PermaNet 2.0[®], PermaNet 3.0[®], LifeNet[®], Olyset Net[®], and Olyset Plus[®] LLINs were used in this study with an untreated net as a control. Six experimental huts were used to randomly allocate one LLIN per hut and allow LLINs to be rotated at each collection. After the first collection, sleepers were randomly allocated to each hut. During the following nights, the sleepers were rotated to avoid confounding factors, such as the sleeper being attractive to the mosquitoes. Collections were conducted from 9:00 PM to 5:00 AM, and indicators were measured according to the protocol of the World Health Organization.

The exophily of *An. gambiae s.l.* was higher with the new generation LLINs Olyset Plus[®] (48.48%) and PermaNet 3.0[®] (78.66%) than with the other LLINs. The only LLIN to induce exophily of *M. Africana* was PermaNet 3.0[®]. LifeNet[®] and Olyset Net[®] were no more effective than the control at reducing the blood feeding rate of *An. gambiae s.l.* ($P>0.05$). However, the new generation of LLINs treated with PBO strongly inhibited blood feeding (61.67% for Olyset Plus[®] and 51.79% for PermaNet 3.0[®]). High mortalities of *M. africana* were observed with LLINs, especially for the Olyset Plus[®] (76.78%) and PermaNet 3.0[®] (95.31%), whereas the mortality rates of *An. gambiae s.l.* were very low: the mortality rate was only 24.68% with PermaNet 3.0[®], which was the most effective LLINs.

This study showed there was a significant heterogeneity in the bio-efficacy of the currently available LLINs against natural populations of *An. gambiae s.l.* in Cove that are resistant to pyrethroids. Both of the LLINs treated with PBO (PermaNet 3.0[®] and Olyset Plus[®]) were more effective against mosquitoes than the other LLINs, which were only treated with pyrethroids.

Keywords: Bio-efficacy, PermaNet3.0[®], Olyset Plus[®], PBO, resistant mosquitoes

Introduction

The fight against malaria in all malaria-endemic areas through mass-distribution campaigns and the high use of Long-Lasting Insecticide-treated Nets (LLINs), Indoor Residual Spray (IRS), and Artemisinin-based Combination Therapies (ACT) have a very good success from 2000 to 2015 [1].

Globally, the declining number of deaths due to malaria was estimated to be 48% with a decline from 839,000 deaths in 2000 to 438,000 in 2015 [1]. Among children less than 5-years-old, the number of malaria deaths decreased from 723,000 in 2000 to 306,000 in 2015 [1].

According to the WHO, the number of deaths in the African region decreased significantly in 2015 [1]. At the same time, the rate of LLIN use increased from less than 2% in 2000 to 68% in 2015 among children under 5-years-old in sub-Saharan Africa [1].

However, the effectiveness of vector control based on insecticides is threatened by mosquito resistance to the insecticides and to IRS (pyrethroids), which are used to treat LLINs [1]. Therefore, to eliminate malaria, it is essential to preserve pyrethroid insecticides as long as possible because no other class of insecticides has equaled the effectiveness, security, cost-efficiency, acceptability, and suitability for LLINs and IRS as pyrethroids [2, 3]. However, current resistance management has a theoretical basis on population genetics that was collected three decades ago [3]. Indeed, a mathematical model simulation showed that the best way to stop the selection of resistance is to use a mixture of different insecticides.

Hence, instead of using a non pyrethroid insecticide to manage resistance, another valid approach for resistance management is the addition of synergists for LLIN treatment. These synergists can reduce resistance by inhibiting the enzymes responsible for resistance. The type of synergist that can inhibit oxidasic resistance involved in the resistance to pyrethroids is piperonyl butoxide (PBO). The aim of this study was to verify the effects of PBO on *Anopheles gambiae s.l.* populations that are resistant to pyrethroids in Benin. This study aimed to compare the effectiveness of two types of LLIN, new generation LLINs (PermaNet 3.0® and Olyset Plus®) and conventional LLINs (PermaNet 2.0®, Olyset Net®, and LifeNet®) in an area high resistance of *Anopheles* to pyrethroids.

The following indicators were used to assess the effectiveness of the LLINs: the penetrating rate of mosquitoes in experimental huts where LLINs and untreated control nets were installed, the exophily rate induced by LLINs on *An. gambiae s.l.* and *M. africana*, the blood feeding inhibition rate, and induced mortality among *An. gambiae s.l.* and *M. africana*.

Methods

Study area and experimental huts

This evaluation was conducted at an experimental station located in a large rice-growing area in Cove, Benin where the resistance level of mosquitoes to pyrethroids was high [4]. The district of Cove is located 159 km from Cotonou, the economic capital of Benin (Figure 1). The climate in Cove is characterized by a rainy season from March to September and a dry season from October to February. Agriculture in Cove is dominated by peanut and rice production.

In the rice-growing area where the experimental station were located, two irrigated rice crops are done each year from March to May and then again from October to January. This practice provides the *Anopheles gambiae s.l.* with breeding sites throughout the year [4]. Seventeen experimental huts have been built on the experimental station using the West African model described in the WHOPES guidelines [4].

Mosquito nets

PermaNet 2.0®, PermaNet 3.0®, LifeNet®, Olyset Net® and Olyset Plus® LLINs were used in this study. PermaNet 2.0® and PermaNet 3.0® were made of polyester fibers coated with

deltamethrin and developed by Vestergaard Frandsen. The difference between these two types of LLINs was that all sides of the PermaNet 2.0® were polyester-coated with deltamethrin while the roof side of the PermaNet 3.0® was made of polyethylene fiber with deltamethrin and incorporated PBO.

The Olyset® net and Olyset Plus® developed by Sumitomo Chemical were made of polyethylene with incorporated Olyset® permethrin. Permethrin + PBO were incorporated in the Olyset Plus®.

The Lifenet® LLIN, which is a product of Bayer CropScience was made of polypropylene fibers coated with deltamethrin.

As a control, an untreated net was used. The LLINs were not washed because the LLINs tested here have already received WHOPES approval.

Mosquito collection

Six experimental huts were used and one LLIN was allocated per hut.

At the first collection, the sleepers were randomly allocated in the huts. During the following nights, the sleepers were rotated using a Latin square design to avoid the particular attraction of mosquitoes to a sleeper. Volunteers slept in the hut from 9:00 PM to 5:00 AM. At 5:00AM, the volunteers dropped the curtain separating the central part of the veranda to prevent mosquitoes from changing compartments. All the dead mosquitoes found inside the huts were collected, and the remaining mosquitoes were collected on the walls and roof of the huts using hemolysis tubes (Figure 2).

Mosquitoes were collected at the place of collection (hut or veranda) and were grouped according to their physiological status (dead or alive, fed or unfed). After morphological identification, the living mosquitoes were provided with a 10% sugar solution and then observed for 24 hours to assess delayed mortality.

At the end of a six-day period, LLINs and other working materials used by the sleepers were rotated following the same Latin square to avoid contamination.

Parameters measured

Four entomological indicators were measured to assess the impact of LLINs on mosquitoes:

Deterrence

The reduction of the penetrating rate of mosquitoes was obtained using the following equation:

$$\text{Reduction rate} = (\text{N control} - \text{N treated}) \times 100 / \text{N control}$$

Where N control is the number of mosquitoes in the control hut, and N treated is the number of mosquitoes in LLIN huts.

Exophily

This measurement was determined by the equation:

$$\text{Exophily rate} = (\text{N veranda}) \times 100 / \text{N Hut}$$

Where N veranda is the number of mosquitoes coming out of the huts to rest on the veranda, and N Hut is the total number of mosquitoes collected inside the hut. For huts provided with LLINs, induced exophily was determined, and for the control hut, natural exophily was determined.

Blood feeding rate

This rate was obtained by a ratio as follows:

$$\text{Blood feeding rate} = (\text{N mosquitoes fed}) \times 100 / \text{total N mosquitoes}$$

Where N mosquitoes fed was the number of mosquitoes fed, and a total N mosquito was the total number of mosquitoes collected.

Lethal effect

The mortality induced by the LLINs was calculated. The following three parameters were evaluated: the immediate mortality, delayed mortality, and total mortality.

Immediate mortality rate = (N immediate dead) x 100 / total N

Delayed mortality rate = (N delayed dead) x 100 / total N

Total mortality = immediate + delayed mortality

Where N immediate dead is the number of mosquitoes that died before the morning, N delayed dead is the number of mosquitoes that died 24 hours post observation time and Total N is the total number of mosquitoes collected.

If the mortality in the control was between 5% and 20%, the mortality in the huts with LLINs were corrected according to Abbott's formula:

Mortality corrected = (E - C) x 100 / (100 - C)

Where E is mortality in the hut with a LLIN and C is the mortality in the control hut.

Side effects of sleeping under the LLINs

The insecticides used for treating LLINs were pyrethroids. These insecticides are not toxic to humans (WHO, 1999). However, any side effects of sleeping under the pyrethroid-treated LLINs that were perceived by the sleepers were collected from the first week until the end of the collection period.

Ethical consideration

This study was approved by the Ministry of Health and the Entomological Research Center of Cotonou. Local adult volunteers who collected the mosquitoes freely gave their consent to participate in the study. They were also subjected to regular medical examinations for preventive treatment of malaria. Moreover, they were vaccinated against yellow fever.

Results

Study of the effectiveness of LLINs

Entry rate of mosquitoes in the boxes provided with treated nets and the witness box

During the evaluation, the following two mosquito species were collected: *Anopheles gambiae s.l.*, the main vector of malaria, and *Mansonia africana*, which is a high nuisance mosquito in the study area. In total, 3,973 mosquitoes were collected: 44.45% were *An. gambiae s.l.* and 55.55% were *M. africana* (55.55%) (Table 1).

Of the five types of LLIN that were tested (PermaNet 3.0[®], PermaNet 2.0[®], LifeNet[®], Olyset[®], and Olyset Plus[®]), none reduced the entry of mosquitoes into the experimental huts when compared to the control.

Exophily of *An. gambiae s.l.* and *Mansonia africana* induced by LLINs

The study of the induced exophily rate showed that pyrethroids used to treat LLINs demonstrated a repulsive effect. The absence of a repulsive effect related to the massive entry of mosquitoes into the huts is probably due to the distance between the location of the huts and the mosquito breeding sites. Mosquito nets treated with synergists (PBO) showed the highest rate of exophily. Overall, half (48.48%) of *An. gambiae s.l.* that entered the Olyset Plus[®] huts were collected in the veranda trap (Table 2). For PermaNet 3.0[®] LLIN, the exophily rate was higher (78.66%). However the repellent effects of PermaNet 2.0[®], Olyset Net[®], Olyset Plus[®], and LifeNet[®] LLINs were not observed for *M. africana*. However, PermaNet 3.0[®] was repellent. Indeed, 44.35% *M. africana* mosquitoes returned to the veranda trap after entering the huts where the PermaNet 3.0[®] LLIN was installed.

Inhibition of gorging by *An. gambiae s.l.* and *Mansonia africana* induced by LLINs

A total of 1,766 *An. gambiae s.l.* were collected in 6 experimental huts during the evaluation, of which 296 fed on blood (16.76%) (Table 3). The blood feeding rate was not the same from one hut to another. The rate was 3.06% in the control hut. No differences in the feeding rate were observed between the control and the LifeNet[®] and Olyset Net[®] LLINs. However, the two new generation LLINs treated with PBO strongly inhibited *An. gambiae s.l.* from blood feeding: PermaNet 3.0[®] by 51.79% and Olyset Plus[®] by 61.67%. However, all the treated nets strongly inhibited *M. africana* from blood feeding (Table 3).

Mortality rate of *An. gambiae s.l.* and *Mansonia africana* induced by LLINs

A high mortality rate was recorded for *M. africana* in this assessment, particularly in huts with PermaNet 3.0[®] (95.31% mortality) and Olyset Plus[®] (76.78% mortality) LLINs (Table 4). However, the mortality rate of *An. gambiae s.l.* was low. These observations suggest that *M. africana* is likely susceptible to pyrethroids and that the lethal effect of LLINs against resistant mosquitoes is low.

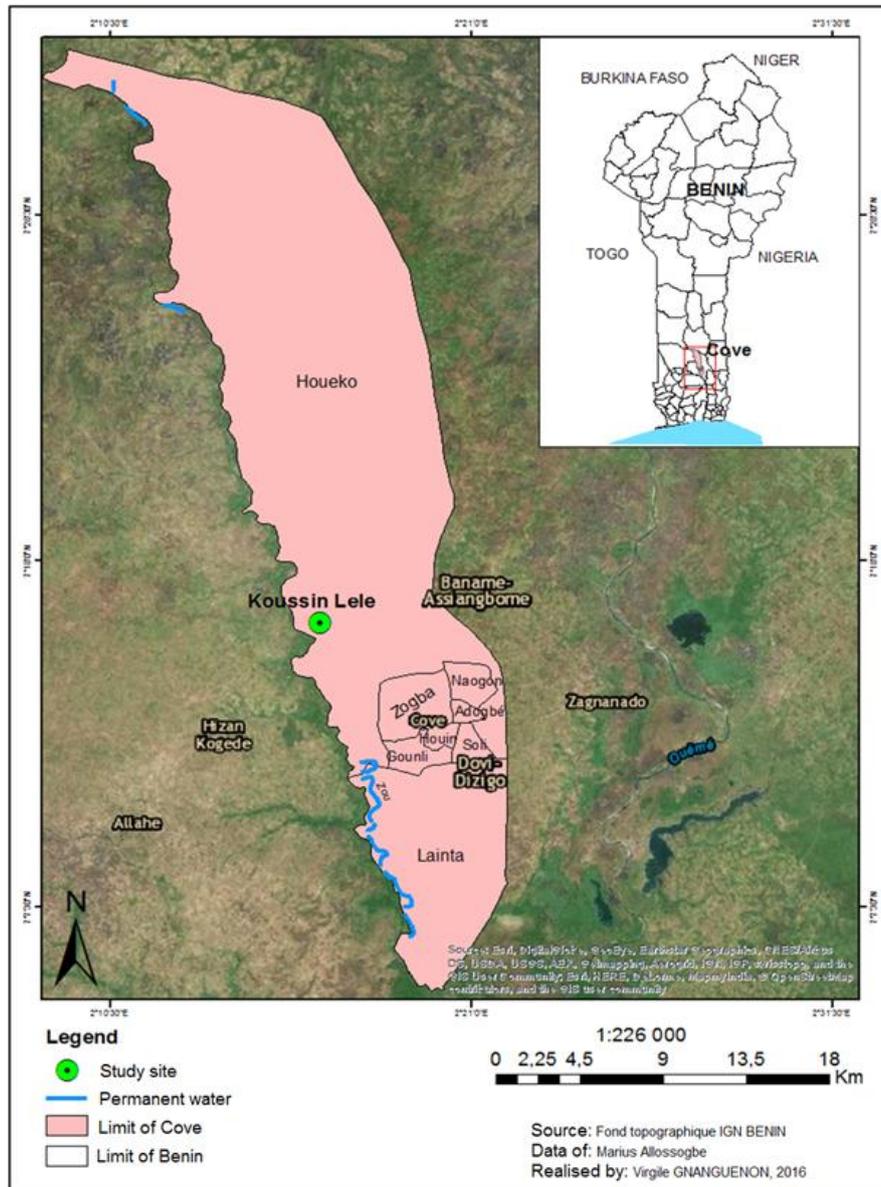


Fig 1: Map of the studied area



Fig 2: Mosquito collection in experimental huts

Table 1: Reduction rate of *An. gambiae s.l.* and *M. africana* induced by LLINs.

LLINs	Mosquitoes collected							
	<i>An. gambiae s.l.</i>				<i>M. africana</i>			
	Total	Hut	Veranda	Proportion (%)	Total	Hut	Veranda	Proportion (%)
Untreated control	243	122	121	-	505	203	302	-
PermaNet 3.0	224	24	200	7.82	456	102	354	9.70
PermaNet 2.0	249	94	155	-2.47	356	108	248	29.50
LifeNet	362	156	206	-48.97	438	160	278	13.27
Olyset Plus	317	82	235	-30.45	237	134	103	53.07
Olyset Net	371	118	253	-52.67	215	110	105	57.43

Table 2: Exophily rate of *An. gambiae s.l.* and *M. africana* induced by LLINs.

LLINs	Mosquito caught									
	<i>An. gambiae</i>					<i>M. africana</i>				
	Total	Hut	Veranda	Proportion (%)	P	Total	Hut	Veranda	Proportion (%)	P
Untreated control	243	122	121	-		505	203	302	-	
PermaNet 3.0	224	24	200	78.66	<0.001	456	102	354	44.35	<0.001
PermaNet 2.0	249	94	155	24.81	0.01	356	108	248	24.53	<0.001
LifeNet	362	156	206	NS	0.09	438	160	278	NS	0.25
Olyset Plus	317	82	235	48.48	<0.001	237	134	103	-40.65	<0.001
Olyset	371	118	253	36.65	<0.001	215	110	105	-27.28	0.01

Table 3: Blood feeding inhibition rate of *An. gambiae s.l.* and *M. africana* induced by LLINs.

LLINs	<i>An. gambiae</i>				<i>M. africana</i>			
	Total	Fed	Inhibition rate (%)	P-value	Total	Fed	Inhibition rate (%)	P
Untreated control	243	54	-		505	63	-	
PermaNet 3.0	224	24	51.79	<0.001	456	10	82.42	<0.001
PermaNet 2.0	249	36	34.24	0.03	356	7	84.24	<0.001
LifeNet	362	88	NS	0.55	438	17	68.89	<0.001
Olyset Plus	317	27	61.67	<0.001	237	5	83.09	<0.001
Olyset	371	67	NS	0.20	215	0	100.00	<0.001

Table 4: Overall mortality rate of *An. gambiae s.l.* and *M. africana* induced by LLINs.

LLINs	<i>An. gambiae s.l.</i>			<i>M. africana</i>		
	Total	Mortality (%)	P-value	Total	Mortality (%)	P
Untreated control	243	-		505	-	
PermaNet 3.0	224	24.68	<0.001	456	95.31	<0.001
PermaNet 2.0	249	10.93	<0.001	356	62.73	<0.001
LifeNet	362	3.56	0.13	438	28.35	<0.001
Olyset Plus	317	8.62	<0.001	237	96.99	<0.001
Olyset	371	6.76	0.01	215	76.78	<0.001

Discussion

Mosquito behavior and resistance status are key factors for determining the efficacy of the insecticides used alone or in combination. The present study confirmed this assumption with a high level of effectiveness observed for Olyset Net[®], PermaNet 2.0[®], LifeNet[®], Olyset Plus[®] and PermaNet 3.0[®] against *M. africana* and a lower level of effectiveness observed against *An. gambiae s.l.* Overall, Olyset Plus[®] and PermaNet 3.0[®] LLINs were significantly more effective than the other LLINs, regardless of the mosquito species. PermaNet 3.0 showed high bio-efficacy against both *An. gambiae s.l.* and *M. africana*.

Thus, the results observed in experimental huts and those obtained in the laboratory strongly suggest that OlysetNet[®], LifeNet[®], and PermaNet 2.0[®] were not very effective against *An. gambiae s.l.* in the areas of high resistance to insecticides in Benin [5]. These results confirm the findings of Asidi *et al.* [6], who showed there was low efficacy of LLINs in communities with high resistance to pyrethroids. Therefore, studies should be conducted to accurately predict the impact

of this low efficacy on vector control tools in Benin. However, several studies have shown that LLINs always protect against infectious mosquito bites, despite the insecticide resistance of vector populations [7]. If the integrity of the LLIN remains intact, the LLIN also represents a physical barrier against mosquito bites [8]. In addition, more than 95% of *M. africana* died when exposed to new generation LLINs treated with PBO. This outcome suggested that these types of LLINs can control malarial vectors and nuisance mosquitoes. Unfortunately, the bio-efficacy of Olyset Plus[®] against *An. gambiae s.l.* was lower than PermaNet 3.0[®]. This result probably occurred due to a strong vector resistance to permethrin in the country [9, 10, 11, 12]. Much of this very high resistance to permethrin is a consequence of using this type of insecticide in agriculture [13, 14]. For example, in Benin, the expansion of cotton growing in the past decade led to the intensive and abusive use of insecticides, which mostly included pyrethroids and permethrin and helped to select resistance at a very high level in malaria vectors. Even if deltamethrin was still effective

against *An. gambiae s.l.* resistant populations, it is necessary to predict the evolution of resistance to this insecticide to consider appropriate management strategies. However, the research of new vector control strategies should not be neglected. Combinations of several insecticides may nevertheless be interesting, especially in terms of vector resistance management^[15, 16].

This study also showed that a very high rate of entry into the huts containing LLINs would probably be related to the low excito-repellent effect that manifests in mosquitoes after contact or close proximity with impregnated LLINs. This outcome confirms the results of a study conducted in experimental huts with deltamethrin-treated nets at Yaokoffikro station on the Ivory Coast^[17]. This study showed that LLINs were generating an excito-repulsive effect against resistant vectors that was less than half of that observed for *An. gambiae s.l.* susceptible to pyrethroids. This decrease in pyrethroid effectiveness against resistant mosquitoes increased the mosquito contact time with the LLINs, which should have resulted in a higher mortality. This was not the case in our study because low mortalities were observed for almost all LLINs against *An. gambiae s.l.* The action that reduces the lethal effect of an insecticide is generated by its own excito-repellent effect. This action was highly observed after close contact of vectors with the LLINs, and a significant proportion of vectors were found on the veranda. It is an action that varies from one insecticide to another and may also explain the different variations in the effectiveness of the LLINs that were tested^[18].

These results have important implications in operational vector control strategies in Benin. These results suggest that a targeted distribution of LLINs with high bio-efficacy against resistant vectors in areas with high resistance to pyrethroids could be useful. Our findings also suggest that an integrated management of vector resistance should be seriously considered for the distribution and use of LLINs in Benin.

Conclusion

This study showed a significant variation in the effectiveness of different types of currently available LLINs that were used against natural populations of *An. gambiae s.l.* that are resistant to pyrethroids in Cote d'Ivoire. New generation LLINs treated with piperonyl but oxide (PermaNet 3.0® and Olyset Plus®) demonstrated higher performance than LLINs without PBO. These results suggest that vector control approaches combining different types of strategies based on pyrethroids, especially in LLINs, could help solve the problem of pyrethroid resistance in malaria vectors. This approach would result in an increase in vector mortality and a reduction of selective pressures on vectors. Continued management of vector resistance control programs is necessary for malaria reduction.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

Authors' contributions

Marius Allossogbe and Martin Akogbéto conceived the study. Marius Allossogbe, Virgile Gnanguenon, Fiacre R. Agossa, André Houtoukpe, Rodrigue Anagonou and Martin Akogbéto have participated in the design of the study. Marius Allossogbe, Jacque Zola-Sahossi, Virgile Gnanguenon,

Rodrigue Anagonou and Martin Akogbéto carried out the field activities and the laboratory analyses. Virgile Gnanguenon has contributed to the mapping. Bruno Akinro did statistical analyzes. Marius Allossogbe, Rodrigue Anagonou, Fiacre R. Agossa, Virgile Gnanguenon and Martin Akogbéto drafted the manuscript. Marius Allossogbe, Rodrigue Anagonou, Virgile Gnanguenon, Fiacre R. Agossa and Martin Akogbéto critically revised the manuscript for intellectual content. All authors read and approved the final manuscript

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