Personal Protection with PBO-Pyrethroid Synergist Treated Nets After Two Years of Household Use Against Pyrethroid-Resistant *Anopheles* in Tanzania

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Research

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Abstract

Background: The spread of pyrethroid resistance in malaria vectors threatens the effectiveness of standard long-lasting insecticidal nets (LLIN). Synergist nets combine pyrethroid (Py) and piperonyl-butoxide (PBO) to enhance potency against resistance mediated by mono-oxygenase mechanisms. Our project assessed personal protection of WHO first-in-class PBO-Py treated nets (Olyset Plus) versus standard LLIN (Olyset net) against pyrethroid resistant *Anopheles gambiae* and *An. funestus* in North West Tanzania after 20 months of household use.

Methods: From household survey, 39 standard Olyset net and 39 Olyset Plus houses were selected, physical integrity and hole index (HI) of nets assessed, resting mosquitoes collected from inside nets and from room walls, indoor abundance estimated using CDC light traps and species identified using PCR. Residual bio-efficacy of PBO and standard LLINs was assessed using 30 min cylinder bioassays.

Results: Of 2397 *Anopheles* collected, 8.9% (n=213) were resting inside standard Olyset nets while none were found inside Olyset Plus PBO-Py nets of any HI category. Resting density of blood fed mosquitoes was higher on walls of sleeping rooms with Olyset net compared to Olyset Plus (0.62 vs 0.10, density ratio: 0.03, 95% CI: 0.01-0.13, $p<0.001$). Mosquitoes were found inside Olyset nets of all WHO HI categories but more were collected inside the more damaged (HI≥ 643) nets than in less damaged (HI 0-64) nets (DR: 6.4, 95% CI: 1.1-36.0, p=0.037). In residual bioassay, mortality of *An. gambiae* s.l was higher with Olyset Plus than with Olyset net for new nets (76.8% vs 27.5%) and 20 months' nets (56.8% vs 12.8%); similar trends were observed with *An. funestus*.

Conclusion: The PBO-Py treated net provided improved protection after 20 months of household use, as demonstrated by the higher bioassay mortality and absence of pyrethroid resistant *An. gambiae* s.s. and *An. funestus* collected from inside Olyset Plus, irrespective of hole index category, as compared to Olyset nets.

Background

Long-Lasting Insecticidal nets (LLINs) are the cornerstone of malaria control in Sub-Saharan Africa. The global malaria burden was reduced by 40% between 2000 and 2015, with insecticide-treated nets (ITNs) and LLINs making the largest contribution to control (1). Since 2015 the annual sub-Saharan malaria burden has not fallen any further (2). The reasons for this are complex. While LLINs may provide personal protection for users even after nets become holed due to their insecticidal and excito-repellency effects (3), with the spread of insecticide resistant mosquitoes, it has been shown that more highly resistant phenotypes can penetrate the LLIN holes to feed (4) and be found resting on inner surfaces (5, 6). In such conditions protective efficacy from malaria may be reduced when physical condition of the net deteriorates (7). Following the selection of high level pyrethroid resistance arising from a combination of *kdr* and mono-oxygenase metabolic mechanisms in North West Tanzania, even new intervention with standard LLINs may not reduce malaria transmission substantially (8–10).

In response to the rapid spread of pyrethroid resistance across Africa, the World Health Organization has encouraged manufacturers to develop new types of LLINs that contain active ingredients with new modes of action to address the problem. Olyset Plus is a new generation LLIN which incorporates the pyrethroid permethrin and synergist piperonyl butoxide (PBO) to counter resistance to pyrethroids in mosquitoes caused by mono-oxygenase metabolic mechanisms (11). PBO inhibits the P450 enzymes which are responsible for detoxification of the pyrethroid before the neuro-toxin reacts with its target site. In a cluster randomized trial conducted in the same area as the present study, full coverage with PBO-Py ITNs (Olyset Plus) provided community protection against malaria up to 2 years of use (12). A further study, this one in Uganda, showed higher efficacy of PBO-Py ITNs compared to standard LLIN over the 18 months duration of the trial (13).

The aim of this study was to examine in an area of high pyrethroid resistance in North West Tanzania the relationship between net holes and blood feeding of mosquitoes collected from inside pyrethroid-only and PBO-pyrethroid nets and from room surfaces containing these nets. The study provides evidence for differences in mosquito feeding success between Olyset Plus and standard Olyset net under household conditions that serves as a proxy for personal protection from Olyset Plus after 20 months of use.

Methods

Study area

This study was embedded within a cluster randomized trial whose main aim was to assess the community effect on malaria of a PBO-Py ITNs (Olyset Plus), in comparison with a standard pyrethroid LLIN (Olyset net), over 3 years of use (12). The present study was conducted 20 months after the distribution of Olyset net and Olyset Plus between October 2016 and February 2017. The data collection was from four villages in Muleba district; Kyamyorwa and Ntungamo received Olyset Plus, Kakoma and Kabirizi received Olyset nets. Details of the study area have been reported previously (14). In brief, the main malaria vectors in Muleba were *An. gambiae* s.s. (92%), *An. funestus* s.l. (4%) and *An. arabiensis* (4%) (12). Pyrethroid resistance was of high intensity, and resistance frequency was over 90% in *An. gambiae* s.l and 45% in *An. funestus* s.l exposed to the WHO diagnostic dose of permethrin (0.75%).

Household and net survey

Ten to twelve houses from each of the four villages were randomly selected, and from each house 1-2 study nets in use were selected at random until 40 nets of each type were reached. Information was collected from each house on household characteristics, LLIN ownership and usage. The selected LLINs were fitted over an Ifakara-style frame (15) and examined for presence, number, position and size of holes in accordance with the WHO LLIN guidelines (16). Holes were categorized into four size classes to estimate the hole area and hole index (HI) of each net. HI was then allocated into three categories: “good” condition (HI 0-64), “acceptable” (65-642), or “torn” (≥643) (17).

Mosquito collection

Repeated mosquito collections were undertaken in each house during 6-10 visits over 5 months to obtain sufficient data for statistical analysis. On each visit, resting mosquitoes were collected from inside the selected study nets, walls of rooms where the nets were installed were searched for mosquitoes using mouth and prokopack aspirators, and CDC light traps were installed for two nights next to the selected nets to collect free-flying mosquitoes. All mosquitoes collected were identified to species (18) and categorised to gonotrophic status. *An. gambiae* s.l. was further identified to species using a real time PCR Taq Man assay to distinguish *An. gambiae* s.s. from *An. arabiensis* (19).

Insecticide bioefficacy of the LLINs

Residual bioefficacy of Olyset nets and Olyset Plus collected after 20 months of use were undertaken on wild-caught *An. funestus* s.l and *An. gambiae* s.l using WHO cylinder test kits. Twenty mosquitoes were exposed to net samples in the WHO cylinders for 30 minutes and mortality recorded 24 hours later (20). Bioefficacy tests were also carried out on unused unwashed Olyset Plus and Olyset nets.

Data analysis

All data analysis was done using Stata version 13. Household characteristics, building materials, presence/absence of eaves, mosquito abundance, hole index and hole area were summarized according to net type and age, adjusting for village and date.

Negative binomial regression was used to estimate the association between hole index and density of blood fed *Anopheles* mosquitoes, adjusting for date and clustering by mosquitoes per net and repeated measures using robust standard errors.

Results

Household and net characteristics

The net surveys were carried out in 20 households with standard Olyset nets and 23 households with Olyset Plus nets. A total of 39 nets of each type were examined. Household and net characteristics were similar in the two groups. Average family size was 6.3 (standard deviation (sd): 1.8) in Olyset net households and 7 (sd: 2.6) in Olyset Plus households. In Olyset net villages, 15 (75.0%, 95%CI: 51.1–89.6) of selected houses had mud walls and 11 (55% 95%CI 32.8–75.4) had open eaves while in Olyset Plus villages 22 (96.7%, 95%CI: 73.2–99.4) had mud walls and 13 (56.5%, 95%CI: 34.8–76.0) had open eaves. Over 80% of each net type had been in use for more than 20 months. Median hole index and hole area in 20 months nets were similar between both types of net (Table 1).

Table 1
Insecticide treated net characteristics and age in months when sampled

	Olyset net		**Olyset Plus**	
	0–5 months	20–21 months	0–5 months	20–21 months
Nets with at least one hole %, (n/N)	33% (2/6)	100% (33/33)	0% (0/4)	94% (33/35)
Hole area (cm^2)				
Mean (std. dev.)	190 (417)	874 (1237)	-	1526 (2689)
Median (IQR)	-	573 (65-1104)	-	640 (95-1562)
Hole index				
Mean (std)	155 (340)	712 (1008)	-	1243 (2191)
Median (IQR)	-	467 (53–898)	-	521 (77-1273)
Hole Index Category % (n)				
0–64	67% (4)	30% (10)	100% (4)	20% (7)
65–642	17% (1)	33% (11)	0% (0)	31% (11)
≥ 643	17% (1)	36% (12)	0% (0)	49% (17)

Mosquito densities according to net type and collection method

A total of 2397 Anopheline mosquitoes were collected from standard Olyset net and Olyset Plus (Table 2). Higher numbers of mosquitoes were collected from households with standard Olyset nets (1631) than with Olyset Plus (766).

Table 2
Indoor density of *Anopheles* mosquitoes according to collection method and net type

	Olyset Net			Olyset Plus		
	Light trap	On Wall	Inside Net	Light trap	On Wall	Inside Net
Total number of collection events	40	240	239	41	300	300
Mean no. Anophelines per collection (N)	17.5 (699)	3.0 (719)	0.9 (213)	6.4 (264)	1.7 (502)	0
Proportion *An. gambiae* s.l. (n)	56.7 (396)	54.7 (393)	61.5 (131)	86.7 (255)	98.7 (465)	0
Proportion *An. funestus* (n)	43.3 (303)	45.3 (326)	38.5 (82)	13.3 (39)	1.3 (6)	0
Total *An. gambiae* s.l. tested for species		175	138		126	0
Proportion *An.arabiensis* (n)		1.1% (2)	0.7% (1)		41.3% (52)	0

N = Total *Anopheles* collected

For standard Olyset nets, a mean of 0.9 mosquitoes were collected per net, 3 mosquitoes per room from wall collection and 17.5 per light trap/night. Overall, 13.1% (n = 213) were collected from inside standard Olyset nets, 42.9% (n = 699) from CDC light traps and 44.1% (n = 719) from the room walls (Table 2). Among the 213 mosquitoes collected from inside the Olyset nets, 91% (n = 193) were freshly blood fed.

Zero mosquitoes were collected from inside Olyset Plus nets (Table 2). Mean number of blood-fed *Anopheles* resting on the walls was also much lower in rooms with Olyset Plus (mean: 0.10) compared to Olyset net (mean: 0.63, DR: 0.16, 95%CI: 0.04–0.69, p-value = 0.015). The two parameters are both indicative of high levels of personal protection.

The overall proportion of *An. funestus* collected relative to *An. gambiae* s.l was much less in villages and houses with Olyset Plus (5.9%, 45/766) than in houses with standard Olyset net (43.6%, 711/1631). Among the *An. gambiae* s.l resting on the wall identified to species, a much higher proportion of *An. arabiensis* (41.6%, n = 52) relative to *An. gambiae* s.s. were found in households with Olyset Plus than in households with standard Olyset nets (0.96%, n = 3).

Blood fed *Anopheles* mosquitoes collected resting from inside nets and on walls.

The average number of *Anopheles* mosquitoes found inside 20 months old Olyset nets was higher in torn LLINs with hole size ≥ 643 than inside standard Olyset nets in “good” condition (hole size 0–64: DR: 6.38, 95% CI: 1.11-36.0, p-value: 0.037) after adjusting for collection date (Table 3). No significant differences in density was found between nets in good and acceptable conditions. Most notably, no mosquitoes were found resting in Olyset Plus nets irrespective of net condition (Table 2). Density of blood fed mosquitoes resting on room walls increased as hole index increased in rooms containing Olyset net, while resting density remained stable across the three HI categories in Olyset Plus rooms (Table 3).

Table 3
Mean density of blood fed *Anopheles* found inside Olyset net and resting on the walls of Olyset net and Olyset plus rooms (> = 20 months) by hole categories

Hole index category	**Total collection**	**Mean *Anopheles* inside net**	**DR***	**95%CI**	**p-value**	**Mean *Anopheles* resting on wall**	**DR***	**95%CI**	**p-value**
Olyset net									
0–64	60	0.5	1			0.05	1		
65–642	66	0.2	0.43	0.06–3.30	0.421	0.23	4.39	0.51–37.8	0.178
≥ 643	72	2.0	6.38	1.11-36.0	0.037	1.68	39.18	6.33–242.5	< 0.001
Olyset Plus									
0–64	54	0				0.09	1		
65–642	90	0				0.10	1.14	0.37–3.47	0.819
≥ 643	126	0				0.12	1.97	0.56–6.91	0.289

* Density Ratio (DR) adjusted for collection date

PBO-pyrethroid ITN bio-efficacy monitoring

Cylinder bioassays with wild, field-caught mosquitoes exposed to Olyset nets showed mortality on new nets of 27.5% in *An. gambiae* s.l and 27.2% in *An. funestus* (Table 4). After 20 months of use the bioassay mortality decreased to 12.8% in *An. gambiae* s.l and to 2.3% in *An. funestus.* The percentage mortality on exposure to new unwashed Olyset Plus was 76.8% in *An. gambiae* s.l and 81.1% in *An. funestus*, and after 20 months of use the mortality decreased to 56.8% in *An. gambiae* s.l and 25.3% in *An. funestus.*

Table 4
Mortality of wild *An. gambiae* s.l. and *An. funestus* after 30-minute exposure and 24 hours holding with new and 20 months Olyset net and Olyset Plus in WHO cylinder bioassays

	Olyset net		**Olyset plus**	
	0 month	20 months	0 month	20 months
An. gambiae				
Total tested	80	78	82	81
% mortality at 24 hours	27.5%	12.8%	76.8%	56.8%
95% CI	17.3–37.7	10.4–15.3	67.6–86.1	46.9–66.6
An. funestus				
Total tested	92	88	95	91
% mortality at 24 hours	27.2%	2.3%	81.1%	25.3%
95% CI	22.5–31.8	0-5.1	65.2–96.9	8.7–41.9
No mortality was observed after exposure to untreated nets in *An. gambiae* (tested = 79) and *An. funestus* (tested = 91)				

Discussion

The study showed that after 20 months of field use, the PBO-Py ITN (Olyset Plus) continued to provide improved personal protection compared to the standard pyrethroid LLIN (Olyset net). There were no mosquitoes to be found resting inside Olyset Plus nets irrespective of LLIN condition or hole index while inside Olyset nets blood-fed *Anopheles* mosquitoes were found resting in nets of all physical conditions, increasing to a mean of 2 *Anopheles* per net in those of the highest hole index category. According to WHO, a hole index that exceeds category HI 643 is considered unserviceable or non-protective in a standard pyrethroid LLIN (17). The presence of blood-fed mosquitoes in standard pyrethroid LLIN with hole index less than this upper limit (i.e. across the range of HI 0-642 rather than just ≥ 643) indicates that the categorization of hole index adopted by WHO to distinguish between serviceable and non-serviceable categories has lost validity with respect to standard LLIN in environments that include a high proportion of highly-resistant vectors. Similarly, in an area of high pyrethroid resistance in Kenya, mosquitoes were collected inside standard LLIN in good and acceptable conditions, while none were found inside standard nets in an adjacent area comprised of susceptible mosquitoes (6).

The findings of the present study are also consistent with the observations made in the cluster randomised trial (CRT), conducted in the same locality, in which no decrease in malaria prevalence in the standard LLIN arm over the first two years of the trial whereas in the PBO-Py ITN arm malaria prevalence decreased by almost one-half during the first two years (12). The complete absence of mosquitoes in the Olyset Plus nets of any hole index category, and low frequency of blood-fed mosquitoes on walls, in the present household study may be interpreted as restoration of protection by the PBO-Py ITN despite the wide range of hole indices to be seen in these nets. This restoration of protection against resistant mosquitoes seems analogous to the historic protection that was achieved when pyrethroid treatment of mosquito nets was first introduced 30 years ago to render untreated mosquito nets more protective against the pyrethroid susceptible vector populations that were prevalent then (3). With respect to PBO-Py ITN which are now being rolled out in preference to standard LLIN in many countries with pyrethroid resistant vectors (21), the HI categorisation and absence of mosquitoes collected inside nets may still serve as a useful predictor of protection elsewhere in Africa until such time that resistance evolves against this net. When that point is reached such highly resistant mosquitoes are predicted to survive and to be found resting inside PBO-Py ITN.

Differences in blood feeding success between Olyset net and Olyset Plus and, by inference, differences in personal protection, have also been observed in pyrethroid resistant areas in experimental hut trials (11, 22). While no contemporary experimental hut trial of Olyset Plus versus standard Olyset net has yet to be reported in Tanzania against a resistant population, experimental hut trials conducted in the pyrethroid resistant West African country of Burkina Faso showed a significantly reduced blood feeding rate in unwashed Olyset Plus compared to Olyset net (22). In Benin, blood feeding rate was higher in hut with Olyset net washed 20 times which is equivalent to 2 years of use compared to Olyset Plus (11). In the same experimental hut trial, it was also shown that the mortality of free-flying resistant mosquitoes induced by Olyset Plus (81%) was double that of Olyset nets (42%) and this ratio did not change significantly after standardized washing over 20 cycles (11). In a meta-analysis of experimental hut studies of several brands of PBO-Py ITN, this class of net was shown to induce 0.60 times less blood feeding than standard pyrethroid-only LLIN and to kill 1.85 times more pyrethroid-resistant mosquitoes (23). In the context of the present Tanzanian study, major differences in mortality in residual bioassay were observed between Olyset Plus and standard Olyset net (~ 50% difference for both *An. gambiae* s.l. and *An. funestus*) after 20 months of use. Of course, differences in mortality is more an indicator of toxicity and reduction of mosquito longevity than of reduced blood feeding rate or personal protection. Nevertheless, the consistency in the trends in West African hut trials and Tanzanian household trial both point towards high efficacy of the PBO-Py ITNs compared to standard LLIN after two years of use.

The protection arising from the interaction between PBO and pyrethroid could be due to inhibition of metabolic resistance by PBO leading to enhanced knockdown and mortality. However, the excito-repellency of mosquitoes away from the net after contact with the PBO-pyrethroid surface may be more pertinent to personal protection than the classical synergising of pyrethroid resistance. For instance, LeClair et al. (14) observed that mosquitoes entering bedrooms were not killed after contact with Olyset Plus but showed increased excitability and a heightened escape reaction and capture rate in light traps present in the same room. More studies would be necessary to unravel the effects of PBO-Py ITNs on mosquito behaviour and synergising of metabolic resistance mechanisms.

The anthropophilic *An. gambiae* s.s was the predominant species, with high proportion of *An. arabiensis* collected from rooms compared to Olyset net rooms. An earlier CRT in this area, reported limited involvement of *An. arabiensis* in malaria transmission based on its sporozoite rate and EIR (24). Previously, in response to the successful control of the primary vector *An. gambiae* s.s. in South Eastern Tanzania following increased coverage of standard LLIN in the universal coverage campaign (25), the ratio of *An. arabiensis* to *An. gambiae* s.s. shifted towards higher *An. arabiensis*. That species shift has been attributed in part to the more zoophilic *An. arabiensis* being less effectively controlled by standard LLIN than *An. gambiae* s.s. in sympatric populations of the species complex (26). In the present study the control of *An. funestus* and, to a lesser extent, of *An. gambiae* s.s. at both community and household levels by PBO-Py ITN (and the failure to control these species in villages and households of the standard LLIN arm) may indicate the beginning of a species shift from *An. funestus* and *An. gambiae* s.s. to *An. arabiensis* in Olyset

Plus villages in NW Tanzania. However, no firm conclusion can be drawn due to the lack of data about species composition resting on the wall in these 4 villages at baseline.

In the present study mortality rates of *An. gambiae* s.l. and *An. funestus* in Olyset Plus bioassays declined after 20 months of use. Despite this, the PBO-Py nets were still protective against mosquito feeding under field conditions.

Conclusion

The PBO-Py ITN Olyset Plus provided improved protection after 20 months of household use, irrespective of hole index, as demonstrated by higher bioassay mortality and the absence of pyrethroid resistant *An. gambiae* s.s. and *An. funestus* collected from inside Olyset Plus as compared to collections from inside standard LLIN.

Declarations

Ethics approval and consent to participate

Written consent was obtained from household head to participate in the study. Ethical approval for the main study was obtained from the ethical review committee of the Tanzanian Medical Research Coordinating Committee (registration number NIMR/HQ/R.8a/Vol IX/1803) and the LSHTM. Further approval was obtained from Ethical committee of Kilimanjaro Christian Medical University College.

Consent for publication

Not applicable

Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly but are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JLM drafted the study protocol, implemented and supervised the field activities, performed molecular work and drafted the manuscript; FM contributed in the study design and supported the field activities; JT and

JFW contributed in the analysis and sample size calculations; NP participated in the design of the study, implementation of activities, data analysis and revised the manuscript; EL & JC participated in data collection, supervision and reviewed the manuscript; MR was the trial principal investigator, contributed to data interpretation and was a major contributor to writing and revising the manuscript. All authors read and approved the final manuscript.

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