Efficacy of the PermaNet Dual compared to the Interceptor G2 and the PermaNet 3.0 in experimental huts in Siaya County, western Kenya.

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Article

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Abstract

Pyrethroid-chlorfenapyr nets have shown significant epidemiological impact over pyrethroid-only and pyrethroid plus piperonal-butoxide (PBO) in Africa. Here we describe a non-inferiority evaluation of PermaNet Dual, a new chlorfenapyr plus deltamethrin net, compared to Interceptor G2, in experimental huts in Siaya, Kenya against free-flying pyrethroid-resistant *Anopheles funestus*. Mosquitoes were assessed for blood feeding and then monitored for immediate knockdown 1-hour post collection and delayed mortality after 72 hours. Mortality at 72 hours was 37% for the control net, 56% for PermaNet 3.0, 66% for the Interceptor G2 and 68% for the PermaNet Dual. Blood feeding inhibition was highest with PermaNet 3.0 at 49%, and least with PermaNet Dual at 12%. PermaNet Dual and Interceptor G2 had no significant differences in mortality (OR = 1.10, 95% CI = 1.00–1.20) or blood feeding inhibition (OR = 1.18, 95% CI = 1.04–1.33) and the lower confidence bounds were within the non-inferiority margins. PermaNet Dual was non-inferior to the Interceptor G2 and superior to the PermaNet 3.0 nets in causing mortality but inferior to PermaNet 3.0 in blood feeding inhibition of the vectors. PermaNet Dual met the WHO criteria for non-inferiority to Interceptor G2 and may be considered for deployment for public health use against pyrethroid-resistant *Anopheles* vectors of malaria.

Introduction

Long lasting insecticidal nets (LLINs) have contributed significantly to the decline in malaria transmission over the past two decades and remain the most widely used malaria vector control tool [1, 2]. LLINs provide a physical barrier against mosquito bites in addition to a toxic dose of insecticides which irritate, repel, knock down and kill the mosquito resulting in reductions in blood feeding and overall mosquito abundance [3, 4]. These chemical properties are particularly useful as the LLIN ages and becomes holed [5]. Insecticide resistance threatens the effectiveness of these vector control tools [6, 7] and for this reason, there is a need for continuous innovation to ensure long lasting insecticidal nets (LLINs) products remain effective against resistant mosquitoes.

Some of the approaches to mitigate insecticide resistance include the use of LLINs treated with a pyrethroid plus a synergist which is not directly toxic to mosquitoes but inhibits detoxification enzymes and restores susceptibility to insecticides. In September 2017, the WHO Global Malaria Programme released updated policy recommendations on the deployment of pyrethroid-PBO (piperonyl butoxide) LLINs [6] followed by the recommendation for deployment of PBO LLINs in areas of ongoing malaria transmission where the principal malaria vector(s) have developed pyrethroid resistance [7]. This recommendation was based on epidemiological data from cluster randomised control trials conducted with Olyset Plus in Tanzania which demonstrated that PBO LLINs have additional public health value [8]. Additional evaluations of pyrethroid PBO LLINs contributed further evidence of efficacy in the following years [9–12].

Pyrethroid-PBO LLINs have faced three main challenges: First, PBO is a synergist for P450 monooxygenases, but insecticide resistance is often as a result of additional mechanisms including

metabolic, target-site [13], cuticular [14] and microbial factors [15]. Second, the PBO incorporated in some of these LLINs was observed to wane in concentration by 18–24 months, well before the expected lifetime of an LLIN which is assumed to be 36 months [16]. Third, the deployment of pyrethroid-PBO LLINs alongside IRS with organophosphates is potentially counterproductive as the P450 monooxygenases also serve to activate organophosphates into their toxic metabolites [17].

More recently, studies have demonstrated the additional benefits of dual active LLINs which incorporate pyrethroid and non-pyrethroid insecticides in the same net. WHO has recently recommended two new classes of LLINs which combine pyrethroids and pyrroles such as chlorfenapyr and pyrethroids and insect growth regulators such as pyriproxyfen (PPF) (https://www.who.int) [18] based on epidemiological impact. Chlorfenapyr acts by disruption of the cellular respiration and oxidative respiration phosphorylation in mitochondria [19]. Its unique mode of action has potential for the control of pyrethroid resistant mosquitoes [20, 21]. The Interceptor G2 is a pyrethroid-chlorfenapyr ITN developed by BASF which has demonstrated improved control of pyrethroid resistant malaria vectors in experimental hut trials in Benin, Burkina Faso and Tanzania [22–24]. Large-scale trials have also provided further evidence of epidemiological impact [25, 26]. Data from experimental hut studies are useful in comparing new products to first in class products that have epidemiological data supporting their use in non-inferiority trials [27]. This study evaluated the non-inferiority of PermaNet Dual, a new pyrethroid-chlorfenapyr LLIN containing deltamethrin and chlorfenapyr against Interceptor G2, a pyrethroid-chlorfenapyr ITN containing alphacypermethrin and chlorfenapyr as well as the superiority of PermaNet Dual over the PermaNet 3.0 which contains deltamethrin and PBO.

Methodology

Study site and design

This study was conducted in experimental huts located on the shores of Lake Kanyaboli (0°02'08.5"N 34°11'05.0"E) in Siaya County, western Kenya. The swamp provides conducive larval habitats for malaria vectors with the most abundant being *An. funestus* followed by *An. arabiensis* with household densities averaging 300 and 20 females per night respectively during peak seasons. Given the low density of *An. arabiensis* during the trial period, only the *An. funestus* densities were analyzed for the experimental hut evaluations.

Seven experimental huts, measuring 6.5 m long × 3.5 m wide × 2 m high (Fig. 1) were used for this evaluation. The walls of the huts are made of blocks lined with mud and have corrugated iron roofs, typical of local housing. Mosquitoes can enter the huts through 10 cm eave gaps on all sides and are prevented from leaving by wood baffles. Each hut has four windows each fitted with exit traps: two on the front and two on the back of the huts. The huts are surrounded by a water-filled moat to prevent the entry of ants and other crawling insects. The floors of the huts are tiled white, for ease of cleaning and collection of dead mosquitoes.

Baseline evaluation of insecticide resistance profile

We conducted both WHO tube and bottle bioassays to assess the intensity of insecticide resistance to pyrethroids in *An. arabiensis* and *An. funestus* vector populations using deltamethrin, permethrin and alphacypermethrin (1x, 2x, 5x and 10x) and deltamethrin + PBO, permethrin + PBO and alphacypermethrin + PBO bioassays. In addition to the pyrethroids, we assessed the susceptibility of the local malaria vectors to pirimiphos-methyl, clothianidin and chlorfenapyr insecticides.

Net treatments and treatment arms

Both PermaNet 3.0 and PermaNet Dual were supplied by Vestergaard Sarl (Lausanne, Switzerland). Interceptor G2 was supplied by BASF (Ludwigshafen, Germany*)*. The untreated nets were made of polyester fabric without any insecticide treatment. The Interceptor G2 was made of polyester fabric coated with 2.4 g/kg (100 mg/m^2) of alphacypermethrin and 4.8 g/kg (200 mg/m^2) of chlorfenapyr. PermaNet 3.0 was made of polyester fabric coated with 2.1 g/kg (84 mg/m^2) of deltamethrin on the sides, and polyethylene incorporated with 4.0 g/kg (120 mg/m^2) of deltamethrin and 25.0 g/kg (800 mg/m^2) of PBO on the roof. PermaNet Dual was made of polyester fabric coated with chlorfenapyr at 5.0 g/kg (200 mg/m^2), and deltamethrin at 2.1kg (84 mg/m^2).

Net Washing

For each study arm, seven nets were randomly selected from a cohort of 21 nets of each production batch and subjected to twenty washes following the WHO washing criteria [28]. To prevent contamination between different types of nets, each net type was washed separately in its own washing station equipped with its separate assortments. The washing process involved immersing each net individually in a 16-L aluminum basin filled with 10 liters of clean groundwater (pH of 7.0 and a hardness of 5 degrees) to which 20g of soap was added and fully dissolved just before washing. Each net was washed for 10 minutes with agitation for 3 minutes, then soaked for 4 minutes and stirred again for 3 minutes. The net samples were rinsed twice in 10L of clean groundwater using the same washing procedure then dried under shade and stored at ambient temperature between washes.

Hut trial procedure

Sleepers rotated between the experimental huts daily while treatments were rotated between the experimental huts on a weekly basis using randomized 7 x 7-Latin square design to adjust for any variation in hut attractiveness. Replicate nets were replaced in the assigned hut daily over the 7-day period. The seven treatments included (1) unwashed polyester untreated net (negative control), (2) PermaNet Dual unwashed, (3) PermaNet Dual washed 20 times (candidate net), (4) PermaNet 3.0 unwashed, (5) PermaNet 3.0 washed 20 times (positive control), (6) Interceptor G2 unwashed and (7) Interceptor G2 washed 20 times (reference net). Six holes, each measuring 4cm x 4cm, were created on every mosquito net, following the WHO guidelines for evaluating LLINs [28]. The long side of the net had two holes at the head section of the sleeper, positioned equidistant to each other. On the other hand, the

short side had only one hole, located at the centre. Between rotations of treatments, the huts were thoroughly cleaned and aired for one day to minimize the risk of cross-contamination.

Mosquito collection

Each night, one person slept under the net in each hut. The sleepers reported to sleep in the huts at 2030hrs until 0630hrs. Each morning, the sleepers collected all the dead and alive mosquitoes inside the huts, nets and exit traps using mouth aspirators (Fig. 4). The mosquitoes were transferred in clean paper cups labelled by collection location and transported to a field laboratory. In the laboratory, the mosquitoes were sorted by status (alive or dead) and by abdominal condition (blood fed, unfed, gravid, or half-gravid), and identified morphologically to genus and species level using taxonomical keys [29]. Live mosquitoes were provided with *ad libitum* access to 10% sugar-water via a small ball of cotton wool. Knockdown was recorded at 1h and live mosquitoes were held for 72h and delayed mortality was recorded at 24h, 48h and 72h.

Supplementary laboratory bioassays

From each arm, 2 net pieces were drawn from before and after the field trial, and 5 pieces cut out (70 pieces total) for cone bioassays against the susceptible *An. gambiae s.s.* Kisumu strain. Only pieces of PermaNet Dual and Interceptor G2 nets (40 pieces total) were used for tunnel tests with the pyrethroid-resistant *An. funestus* Siaya strain to evaluate the slow acting chlorfenapyr components of PermaNet Dual and Interceptor G2. *Anopheles gambiae s.s.* Kisumu strain is an insecticide-susceptible reference colony that originated from Kisumu, western Kenya. *Anopheles funestus* Siaya strain was the F1 progeny of mosquitoes collected from households around the experimental hut site and are resistant to pyrethroids but susceptible to organophosphates, clothianidin and chlorfenapyr.

Ten three-day-old mosquitoes were exposed to each net piece (30cm by 30 cm) cut from the 5 net sides for 3 minutes in two replicates in cohorts of 5 mosquitoes per cone. After exposure, mosquitoes were transferred to labelled paper cups, provided access to 10% sugar water and held at 27 ± 2°C and 75 ± 10% relative humidity (RH) with knockdown and mortality recorded after one hour and 24 h, respectively, for PermaNet 3.0. In contrast, mortality for PermaNet Dual and Interceptor G2 were monitored every 24h up to 72 h.

Tunnel assays were conducted against the pyrethroid-resistant *An. funestus* Siaya strain with the same net pieces of Interceptor G2 and PermaNet that were tested in the cone assay. The tunnel test chamber mimics the behavioral interactions that occur between free-flying mosquitoes and nets during host seeking. It consists of a square glass tunnel divided one third (20cm) its length by a box frame fitted with a net sample. In the short section of the tunnel, a rabbit bait was held in a cage with its back sheared and exposed for ease of accessibility and feeding by mosquitoes while in the long section (40cm), 100, 5–8 day old mosquitoes were released at 6pm and left until 6am. The net pieces used in the experiment had nine small holes, each measuring 1 cm in diameter, which allowed mosquitoes to enter the baited chamber. In the morning, the mosquitoes were collected from the tunnel and examined for mortality and

blood feeding. The surviving mosquitoes were placed in clean paper cups with a label and given access to a 10% sugar solution. They were kept at a temperature of 27 ± 2°C and a relative humidity of 75 ± 10%. Delayed mortality of the mosquitoes was recorded every 24 hours, up to a maximum of 72 hours.

Chemical Assays

Two nets were randomly selected from all the wash points in every arm, before and after the hut trials, and five pieces were obtained from each net apart from PermaNet 3.0, from which 3 pieces were obtained from the top and 1 from each side (7 pieces total) following WHO guidelines on net cutting [30]. The cut net pieces were shipped wrapped in aluminium foil to the Vestergaard ISO/IEC 17025 accredited Vector Control Laboratories in Vietnam for testing to determine the wash retention of active ingredients in the net pieces using analytical methods validated and published by the Collaborative International Pesticides Analytical Council (CIPAC). Briefly, deltamethrin in the roof of PermaNet 3.0 (roof) was extracted from net samples by heating under reflux for 30 min with xylene using dicyclohexyl phthalate as internal standard. The solvent was evaporated, and the residue dissolved in hexane. Deltamethrin was extracted from the nets, including PermaNet Dual and PermaNet 3.0 sides using dicyclohexyl phthalate and the concentration determined by normal phase high performance liquid chromatography with UV diode array detection (HPLC–DAD). Alphacypermethrin in Interceptor G2 as well chlorfenapyr in Interceptor G2 and PermaNet Dual were sonicated with heptane using dicyclohexyl phthalate as internal standard and determined by gas chromatography with flame ionisation detection (GC-FID). Lastly, PBO in PermaNet 3.0 roof was extracted from net samples by heating under reflux for 30 min with xylene using octadecane as internal standard and determined by GC-FID.

Data analysis

The primary outcomes measured by comparing the treatments and control experimental huts were; blood feeding inhibition (the reduction in blood feeding in treatments compared with that in the control huts), immediate and delayed mortality (the proportion of mosquitoes that are dead in the morning of collection and the cumulative proportion dead at 24, 48 or 72 hours). In addition, we evaluated induced exophily (the proportion of mosquitoes that are found in the exit traps) and deterrence (proportional reduction in the number of mosquitoes collected in the treated huts relative to the number collected in the control huts with untreated nets).

The difference in proportional outcomes (mortality, blood feeding and exophily) between treatments and control at all wash points were analysed using a blocked logistic regression model, while differences in numerical outcomes (entry) was analysed using a negative binomial regression model. Tests of non-inferiority between PermaNet Dual and Interceptor G2 for both mortality and blood feeding were performed according to the WHO protocol [31]. The analysis included both washed and unwashed nets with an independent variable included in the model for washing. A candidate product is considered non-inferior to the active comparator product if: (a) the lower 95% confidence interval of the odds ratio describing the difference in mortality between the candidate and comparator product is > 0.7 and/or, (b) the upper 95% confidence interval of the odds ratio describing the difference in blood feeding between the

candidate and comparator product is < 1.43. The superiority between PermaNet Dual and PermaNet 3.0 was also assessed based on whether mortality rates were higher and blood feeding rates lower at 5% significance level (i.e. $p < 0.05$). All analyses were done using R Statistical Software (v4.2.2; R Core Team 2021).

Ethical Considerations and compliance with GLP

Ethical approval for the trial was issued by Scientific and Ethical Review Unit (SERU) of KEMRI, (SERU 4536). This study was also reviewed by CDC and was determined to meet the definition of research involving human subjects but CDC's involvement was not considered to constitute engagement in human subjects research. Prior to recruitment into the study, a formal informed consent was obtained from the volunteer sleepers. The participants were each provided with a course of weekly prophylaxis over the study period to protect them from contracting malaria. This site is accredited by the Kenya Pest Control Products Board (PCPB) for national evaluation of vector control products for registration purposes. The study was conducted in strict conformance of WHO non-inferiority guidelines for evaluation of second in class LLINs [27, 31]. Additionally, the site has begun the process towards GLP accreditation and conducts all study procedures in strict conformance with GLP requirements.

Results

Insecticide resistance profile of the local mosquito populations

Anopheles funestus was resistant to all three pyrethroid insecticides up to 5X the diagnostic dose. This species was also resistant to permethrin up to 10X the diagnostic dose while it was suspected resistant at the 10X dose for alphacypermethrin and deltamethrin. *Anopheles gambiae* s.l. was resistant to alphacypermethin up to the 5X dose with suspected resistance at the 10X dose and to deltamethrin up to the 5X dose. *Anopheles gambiae* s.l. was susceptible at the 10X dose for deltamethrin. For permethrin, *An. gambiae* was resistant at the 1X dose but susceptible at the 5X and 10X doses (Table 1). Both *An. gambiae* and *An. funestus*, sampled around the experimental huts, exhibited resistance to the highest concentration (10x) of alphacypermethrin insecticide. Pre-exposure to PBO partially or fully restored the susceptibility of both species to the three-pyrethroid insecticides. After PBO pre-exposure, the mortality rate increased to > 95% for both species. Both malaria vector species were 100% susceptible to clothianidin, chlorfenapyr, and pirimiphos methyl.

Table 1
Insecticide resistance status of malaria vectors of Lake Kanyaboli, western Kenya

Assays	Insecticide	Dose	Concentration	Sample size	% Mortality	
					An. gambiae	**An. funestus**
WHO tube	Alphacypermethrin	1X	0.05%	100	82	45
		5X	0.25%	100	88	60
		10X	0.50%	100	93	94
	PBO + Alphacypermethrin	1X	0.05%	100	95	97
	Deltamethrin	1X	0.05%	100	45	77
		5X	0.25%	100	84	72
		10X	0.50%	100	100	92
	PBO + deltamethrin	1X	0.05%	100	100	97
	Permethrin	1X	0.75%	100	82	64
		5X	3.75%	100	98	94
		10X	7.50%	100	100	86
	PBO + permethrin	1X	0.75%	100	99	95
	Pirimiphos-methyl	1X	0.25%	100	100	100
WHO bottle	Clothianidin		4µg/ml	100	100	100
	Chlorfenapyr		100µg/ml	100	100	100

Mosquito entry and exiting rates in experimental huts:

A total of 15,114 pyrethroid resistant female *An. funestus* were collected during the experimental hut evaluation. Significantly more mosquitoes were collected in huts with the unwashed PermaNet 3.0 compared to the washed PermaNet 3.0 and the washed and unwashed Interceptor G2. No other differences in hut entry were observed. Exit rates were significantly higher for the washed and unwashed PermaNet 3.0 compared to all other treatments while the exit rates for the unwashed Interceptor G2 were significantly lower compared to the untreated net. No other significant differences in exit rates were observed. (Table 2).

Table 2
Entry and exiting rates of wild Anopheles funestus in experimental huts in Siaya, Kenya

Net type	Net status	Average females caught/hut/night*	Deterrence (%)	Total exiting	Exophily* (%)	95% CIs
Untreated net	—	45	-	827	37[c]	35–40
PermaNet 3.0	Unwashed	49.6	-10	1347	55[a]	53–57
	Washed 20x	40	11	1122	57[a]	55–59
PermaNet Dual	Unwashed	46.7	-3	610	27[bc]	25–29
	Washed 20x	44.8	1	713	32[bc]	31–35
Interceptor G2	Unwashed	41.9	7	575	28[b]	26–30
	Washed 20x	40.4	10	728	37[bc]	35–39

*Values in the same column bearing the same letter do not differ significantly at the 5% level

Mortality of wild pyrethroid resistant Anopheles funestus in the experimental huts.

Mortality was highest in the unwashed PermaNet Dual (68%) followed by the washed PermaNet Dual (67%). Mortality for the washed and unwashed Interceptor G2 were 66% and 64%, respectively (Fig. 2). Mortality observed in huts with PermaNet Dual and Interceptor G2 were statistically higher than mortality observed in the washed and unwashed PermaNet 3.0 (56% in both) which were significantly higher compared to the untreated net (37%). We observed high mortality in huts with untreated nets which as mostly due to high mortality of mosquitoes collected in exit traps which was likely caused by strong winds emanating from Lake Kanyaboli (Table 3).

Table 3
Mortality of pyrethroid resistant Anopheles funestus in experimental huts

Net type	Net status	Average females caught/hut/night	Total 72h mortality	% 72h mortality*	95% CIs	% Overall killing effect
Untreated net	-	45	817	37[c]	35–39	-
PermaNet 3.0	Unwashed	49.6	1350	56[ab]	54–58	24
	Washed 20x	40	1091	56[a]	53–58	12
PermaNet Dual	Unwashed	46.7	1549	68[b]	66–70	33
	Washed 20x	44.8	1480	67[b]	65–69	30
Interceptor G2	Unwashed	41.9	1319	64[ab]	62–66	23
	Washed 20x	40.4	1310	66[ab]	64–68	22

*Values in the same column bearing the same letter do not differ at the 5% significance level.

Mosquito blood feeding rates in experimental huts

The percentage blood-feeding rate was lowest in huts with the washed PermaNet 3.0 (8%) and highest in unwashed PermaNet Dual and the untreated net (16%) though this was not statistically different from washed PermaNet Dual (14%) (Table 4). There was no difference in blood-feeding proportion in huts with Interceptor G2 (13%) nets for both unwashed and washed.

Table 4
Blood feeding rates of wild Anopheles funestus in experimental huts in Siaya, western Kenya.

Net type	Net status	Average females caught/hut/night	Total blood-fed	% Blood feeding*	95% CIs	% Blood feeding inhibition	% Personal protection
Untreated net	-	45	356	16[b]	15–18	-	-
PermaNet 3.0	Unwashed	49.6	263	11[ab]	10–12	33	26
	Washed 20x	40	160	8[a]	7–9	49	55
PermaNet Dual	Unwashed	46.7	359	16[b]	14–17	3	-1
	Washed 20x	44.8	312	14[b]	13–16	12	12
Interceptor G2	Unwashed	41.9	266	13[ab]	12–14	20	25
	Washed 20x	40.4	251	13[ab]	11–14	21	29

*Values in the same column bearing the same letter do not differ significantly at the 5% level.

Non-inferiority assessment

According to the recent provisional WHO guidelines, for a candidate LLIN to be included in an established intervention class, it must demonstrate non-inferiority to the first in class product which has already demonstrated public health value (Interceptor G2, for pyrethroid-chlorfenapyr ITN class) and superiority to pyrethroid only LLIN in experimental hut trial [31].

The non-inferiority margin is set at 0.7 for mortality and 1.43 for blood feeding. The odds ratio for the difference in mosquito mortality between PermaNet Dual and Interceptor G2 was 1.10 (95% confidence interval: 1.00-1.20) while the odds ratio for the difference in mosquito blood feeding was 1.18 (95% confidence interval: 1.04–1.33) in mosquitoes. Following the WHO criteria described above PermaNet Dual is non-inferior to Interceptor G2 based on the mortality induced in pyrethroid resistant *An. funestus* in the experimental hut trial in Lake Kanyaboli, Kenya while the PermaNet Dual is both inferior and non-inferior to the Interceptor G2 based on blood feeding inhibition (Table 5). For the superiority assessment, PermaNet Dual was superior to PermaNet 3.0 in mortality induced, 68% vs 56%, but was inferior to PermaNet 3.0 in blood feeding (Table 5).

Table 5
Results from the non-inferiority assessment of PermaNet Dual to Interceptor G2 against wild pyrethroid-resistant Anopheles funestus in experimental huts in Siaya western Kenya.

Primary indicators	Variables	Superiority assessment		Non-inferiority assessment	
		PermaNet 3.0	PermaNet Dual	Interceptor G2	PermaNet Dual
	Total collected	4390	4481	4036	4481
Mortality	Total dead	2441	3029	2629	3029
	Mortality (%)	56	68	65	68
	Odds ratio	-	1.805	-	1.096
	Std. error (on log odds scale)	-	0.080	-	0.050
	P-value	-	< 0.001	-	0.047
	95% CIs	-	1.654–1.969	-	1.001–1.199
	WHO efficacy criteria	-	Significantly higher ($p < 0.05$)	-	Lower 95% CI > 0.7
	Conclusion	-	**Superior**	-	**Non-inferior**
Blood feeding	Total blood-fed	423	671	517	671
	Blood-feeding (%)	10	15	13	15
	Odds ratio	-	1.627	-	1.176
	Std. error (on log odds scale)	-	0.110	-	0.076
	P-value	-	< 0.001	-	0.012
	95% CIs	-	1.425–1.856	-	1.037–1.334
	WHO efficacy criteria	-	Significantly lower ($p < 0.05$)	-	Upper 95% CI < 1.43
	Conclusion	-	**Inferior**	-	**Inferior and Non-Inferior**

Supplementary assay results

Both washed and unwashed PermaNet Dual and Interceptor G2 pieces tested induced low mortality in cone bioassays (< 73% for all tests, Fig. 3) against susceptible *An. gambiae s.s*, Kisumu strain, an indication that the cone bioassay is not suitable for testing slow acting actives even when combined with pyrethroids, a fast-acting active ingredient. PermaNet 3.0 roof net pieces induced highest mortality rates (100%) for all the wash points, with sides inducing mortality rates of > 92% (Fig. 3).

Tunnel assays results

Mortality rates of *An. funestus* in tunnel tests against the Interceptor G2 and the PermaNet Dual were high at all wash points (< 96.6%). Interceptor G2 induced the highest mortality rate with 20 washes after the hut trial at 99.1% while PermaNet Dual had highest mortality rate of 98.2% with unwashed net pieces obtained from LLINs pieces after hut trial. However, there was no significant deference in mortality between the two pyrethroid – chlorfenapyr LLINs (Fig. 4).

High blood feeding inhibition of 96% was witnessed with samples of unwashed PermaNet Dual after the hut trial whereas Interceptor G2 washed 20 times pieces after hut trial induced the lowest blood feeding inhibition of 80% (Fig. 5).

Chemical assays

All the unwashed LLINs had AI content within the manufacturer specified range. Retention of AI was lowest in the net pieces cut from the PermaNet Dual washed 20 times (43% deltamethrin and 47% chlorfenapyr) and highest in the net pieces cut from the Interceptor G2 washed 20 times (83.5% alpha-cypermethrin and 81% chlorfenapyr). Net pieces obtained from the PermaNet 3.0 had retention of 64, 92.8 and 82% for deltamethrin on the sides, deltamethrin on the roof and PBO respectively (Table 6).

Table 6
The content of active ingredients contained in unwashed and washed net pieces before and after the experimental hut trial in Siaya, Kenya.

ITN Brand	Active Ingredient (s)	AI content (g/kg)		AI retention (%)
		Unwashed	Washed 20X	
PermaNet 3.0	Deltamethrin (sides)	1.75	1.12	64.0
	Deltamethrin (roof)	3.61	3.35	92.8
	PBO (roof)	19.11	15.69	82.1
Interceptor G2	Alpha-cypermethrin	2.85	2.38	83.5
	Chlorfenapyr	5.56	4.51	81.1
PermaNet Dual	Deltamethrin	2.09	0.90	43.1
	Chlorfenapyr	5.00	2.38	47.6

Discussion

This study evaluated the efficacy (mortality and blood feeding inhibition) and wash resistance of PermaNet Dual (Vestergaard Sarl) in comparison to Interceptor G2 (BASF) and PermaNet 3.0. (Vestergaard Sarl) against pyrethroid resistant free flying *An. funestus* mosquitoes in experimental huts

on the shores of Lake Kanyaboli in Siaya County, western Kenya. This locality has a year-round abundance of *An. funestus* and seasonal abundance of *An. arabiensis*. This trial was conducted in the dry season and therefore only *An. funestus* had adequate numbers for statistical comparisons, averaging 44 female mosquitoes per hut per night. The Lake Kanyaboli area is mostly swampy with permanent stagnated pools of water conducive for the development of *An. funestus* with peak numbers > 300 mosquitoes per structure per night in the rainy seasons (Ochomo *et al.*, unpublished).

All three LLINs evaluated here had significantly higher mortality rates on the free flying *An. funestus* mosquitoes relative to the control in the experimental huts. PermaNet Dual induced the highest mortality rates which was not significantly different from Interceptor G2 but was significantly higher than PermaNet 3.0 at corroborating results from previous hut trials in Benin [32]. Similar observations have been made in experimental hut trials evaluating PermaNet Dual and Interceptor G2 where in each instance, the pyrethroid-chlorfenapyr LLIN induced higher mortality than the pyrethroid-PBO or pyrethroid only LLINs [23, 32, 33].

Despite the lower mortality observed in this and other experimental hut studies, pyrethroid-PBO LLINs have been shown to offer up to 2 years better protection, with reduced parasite prevalence and vector densities than pyrethroid-only LLINs in Uganda and Tanzania [8, 9]. However, the rapid loss of PBO is a concern. A study in Tanzania noted that the PBO content of the nets was significantly reduced at 12 months and was almost lost by 24 months, a risk for sustained efficacy against pyrethroid resistant malaria mosquitoes over the expected three-year lifetime [10]. For this reason, dual active nets with three years of effectiveness are urgently needed to complement vector control efforts in areas of high pyrethroid resistance.

High resistance to alphacypermethrin, deltamethrin and permethrin was observed in both *An. funestus* and *An. arabiensis* which coincides with earlier reports [34, 35]. Higher concentrations of deltamethrin and permethrin in WHO tube assays (0.50% and 7.5% respectively) and deltamethrin and alphacypermethrin (5x and 10x, respectively) in bottle assays were effective against *An. gambiae*, but not against *An. funestus*, indicating higher intensity of resistance in *An. funestus* relative to sympatric vectors. We observed full susceptibility of both malaria vectors from the area to non-pyrethroids insecticides at standard doses: neonicotinoids (clothianidin), pyrrole (chlorfenapyr) and organophosphate (pirimiphos-methyl) despite high resistance to pyrethroids indicating that these classes could be effective for rotation or use of mixture formulations for malaria control in the region. The above finding was also an indication that there was no cross resistance between pyrethroids and these other classes of insecticides. The addition of PBO as a synergist was observed to partially restore the observed susceptibility in both *An. arabiensis* and *An. funestus* indicating the involvement of P450 monooxygenases in the resistant phenotypes as has been reported elsewhere [36–38]. However, it partially restored susceptibility to > 95% mortality, which is close to full susceptibility, but suggests the involvement of other resistance mechanisms.

PermaNet Dual was non-inferior to Interceptor G2 (the first in class), with an odds ratio of 1.10 (1.00-1.20) at a non-inferiority margin of 0.7 according to the WHO guidelines for evaluation of non-inferiority to first in class products [41]. Following this criterion, PermaNet Dual does not need to undergo evaluation for epidemiological impact but is available for recommendation as a second product in the same class. The PermaNet Dual has since been prequalified by WHO (https://extranet.who.int/pqweb/vector-control-product/PermaNet-dual) and is therefore available for immediate deployment to contribute to insecticide resistance management (IRM). Additionally, PermaNet Dual was superior in inducing mortality relative to PermaNet 3.0 with an odds ratio of 1.81 (1.65–1.97). This shows the contribution of chlorfenapyr to the control of resistant mosquitoes where mechanisms other than P450 monooxygenases are active such as in this population. Similar findings have been documented in Tanzania [10], where there was a higher impact on entomological outcomes in clusters with Interceptor G2 than those with PermaNet 3.0, and in another experimental hut trial evaluating the non-inferiority of PermaNet Dual to Interceptor G2[32].

Blood-feeding inhibition was significantly higher with PermaNet 3.0 compared to both Interceptor G2 and PermaNet Dual but was not significantly different between the two pyrethroid-chlorfenapyr nets. Results from a separate study comparing Interceptor G2 and chlorfenapyr only control showed higher blood feeding rates in the chlorfenapyr only arm indicating that pyrethroids contribute the most to blood feeding inhibition [33]. The current study therefore indicates that PBO in PermaNet 3.0 synergized the blood feeding inhibition and therefore lower blood feeding rates were realized compared to the pyrethroid-chlorfenapyr nets. PermaNet 3.0 was therefore superior to PermaNet Dual in blood feeding inhibition. PermaNet Dual was non-inferior to Interceptor G2 in blood feeding inhibition possibly due to the higher irritability of alphacypermethrin.

These results were not significantly different between unwashed nets and nets washed 20X, although the trends were towards higher mortality in the 20X washed nets. PermaNet Dual and Interceptor G2 did not have reductions in induced mortality or blood feeding inhibition after 20 washes, indicating good wash resistance, which is the current standard WHO proxy for an LLIN giving good performance for up to three years of use, despite less than 50% AI retention in the PermaNet Dual. Previous studies have reported similar results [32, 33].

Standard laboratory cone bioassays with PermaNet Dual and Interceptor G2 failed to predict their efficacy against pyrethroid resistant *An. funestus* s.l. in experimental huts. Cone bioassays with pyrethroid - chlorfenapyr nets did not meet the WHO criteria for the susceptible *An. gambiae* s.s. Kisumu strain PermaNet 3.0 while tunnel tests with the PermaNet Dual resulted in > 95% mortality against F1 progeny of wild *An. funestus*, affirming the unsuitability of cone bioassays for the evaluation of chlorfenapyr LLINs. These findings are similar to earlier ones reported in Benin and Cote d' Ivoire [32, 42] and indicate that tunnel tests are required as a laboratory assay of pyrethroid plus chlorfenapyr nets.

The main limitations of the study included the unexpectedly high rate of exophily in control arm which was higher than PermaNet Dual and Interceptor G2 at 28%, and which could not be explained. Mortality at 72 hours in the control arm was 37% which was higher than most other hut studies including another

pyrethroid-chlorfenapyr net experimental hut study with *An. funestus* in Tanzania [31]. This was likely due to excess mortality in the exit traps as the experimental hut sites are located on the shores of Lake Kanyaboli and receive strong winds through the night which desiccated the mosquitoes which escaped into the exit traps leading to increased mortality. However, given the high densities of *An. funestus* per hut per day (44), this did not affect the statistical power of the study.

Conclusions

PermaNet Dual, the candidate product (deltamethrin + chlorfenapyr), was non-inferior to Interceptor G2, the reference product (alphacypermethrin + chlorfenapyr) in causing mortality and inducing blood feeding inhibition of free flying wild pyrethroid resistant *An. funestus* in this experiment. PermaNet Dual was superior to PermaNet 3.0, the positive control (deltamethrin + PBO) in causing mortality but inferior in the blood feeding inhibition of wild pyrethroid resistant *An. funestus* in this experiment. Overall, PermaNet Dual met the WHO efficacy criteria in relation to non-inferiority to Interceptor G2 and can be deployed in areas of high pyrethroid resistance.

Declarations

Consent for publication

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Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the views, decisions, or policies of the U.S. Centers for Disease Control and Prevention.

Competing interests

All the authors declared that they have no competing interests.

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Data availability

All the raw data are available and submitted alongside this manuscript

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Authors contributions

EO and BA conceived the study. NO, SA and EO designed the study and excuted the trial. VM and BA analysed the data. NO, SA wrote the manuscript. CO, ER, LK, JG, and EO revised the manuscript. All authors reviewed and approved the final manuscript for publication.

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Figures

Figure 1

Experimental hut design: A front view of the hut fitted with window exit traps, B showing the wood baffles, and C showing the tiled floor and the hut interior walls.

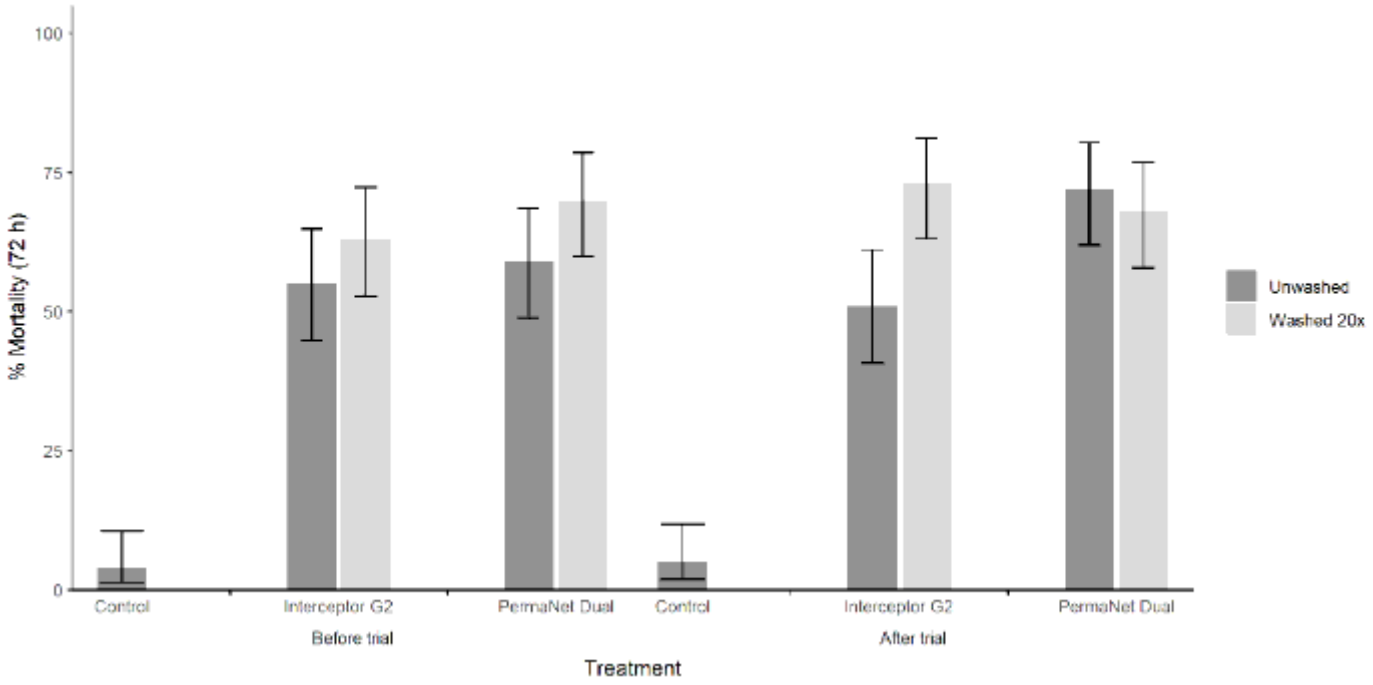

Figure 2

Results of the cone test for Interceptor G2and PermaNet Dual.

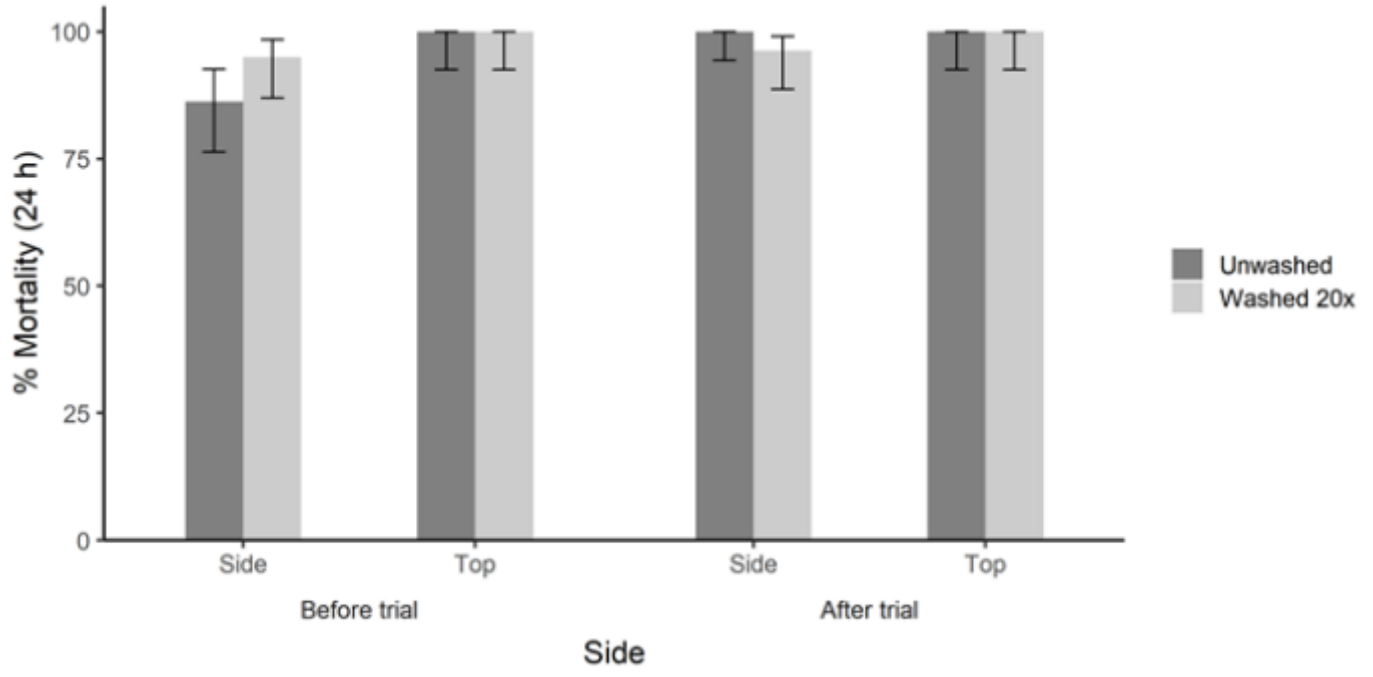

Figure 3

Results of the cone tests for PermaNet 3.0

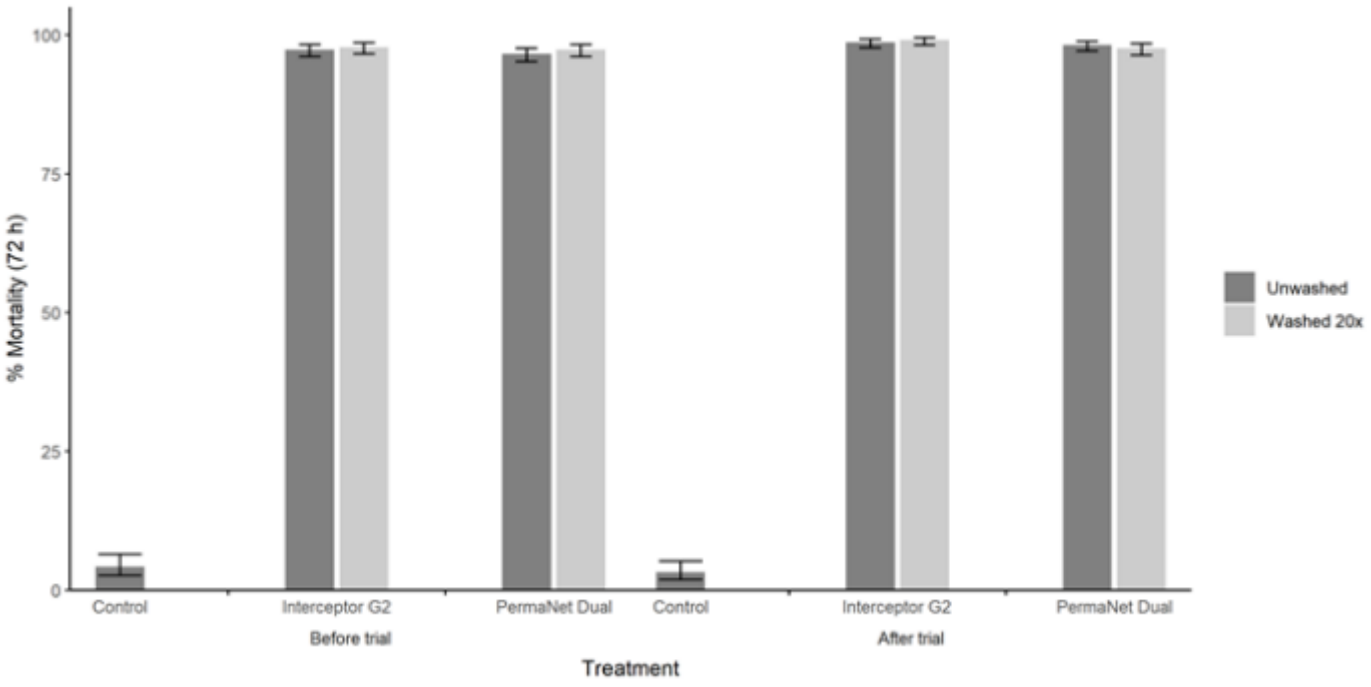

Figure 4

Mortality rate of wild An. funestus F1 mosquitoes exposed to Interceptor G2 and PermaNet Dual in tunnel tests.

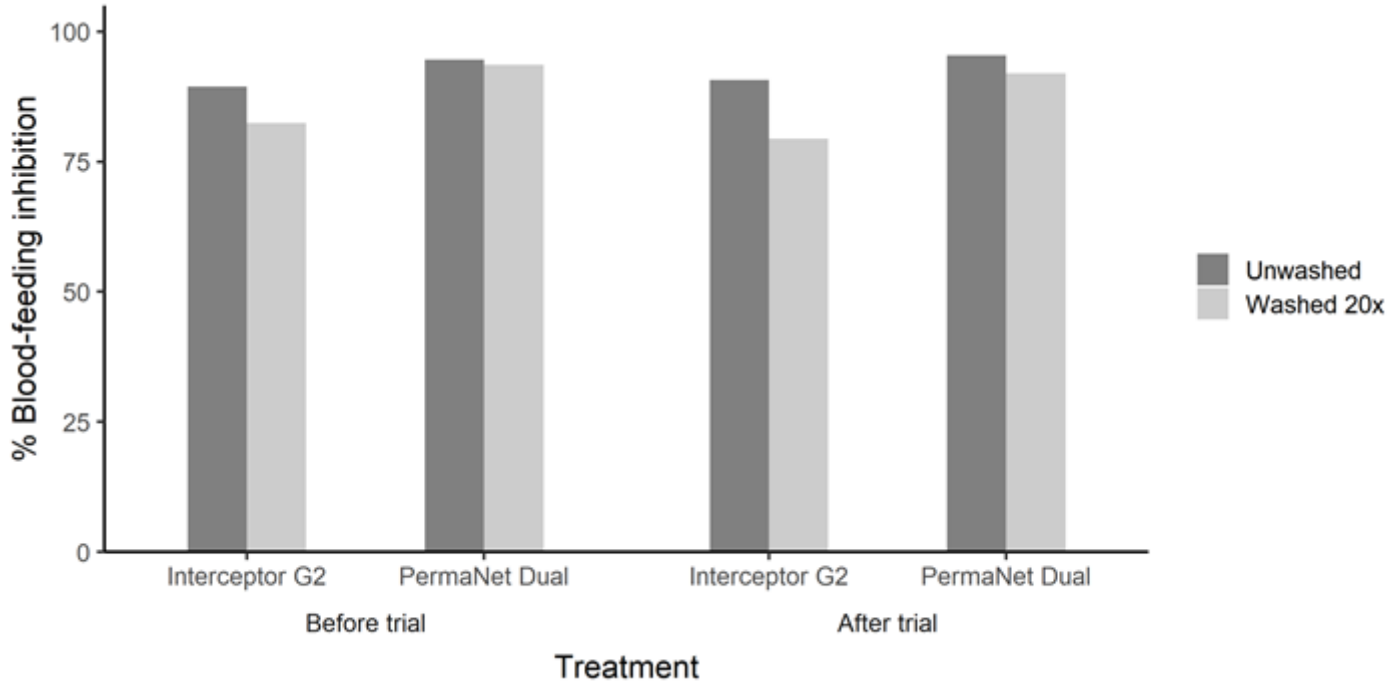

Figure 5

Percentage of blood feeding inhibition by Interceptor G2 and PermaNet Dual in tunnel test.