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RESEARCH

Laboratory evaluation of a new alphacypermethrin long-lasting insecticidal net against *Anopheles culicifacies s.l.*

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Abstract

Long-lasting insecticidal nets (LLINs) are being promoted by World Health Organization (WHO) as key vector control tool for the prevention and control of malaria and other vector-borne diseases. A laboratory study was conducted to evaluate the bio-efficacy of a new alphacypermethrin LN (HILNET®) against insecticide-susceptible *Anopheles culicifacies s.l.*. This study presents the observations on (a) regeneration time, the period required to regenerate insecticide in the LLIN after three consecutive WHO standard washes in a single day to deplete the insecticide on the net surface, and (b) wash resistance of the LLIN to retain bio-efficacy up to 20 repeated washes at intervals corresponding to the regeneration time in days. In the present evaluation, the regeneration time of HILNet® was determined as 2 days. Wash resistance studies of HILNet® showed a knockdown rate of 100% up to 15 washes and 97% at the 20th wash and complied with the criteria of WHO knockdown rate of ≥ 95%. The mean active ingredient content from 0 to 20 washes was in the range of 5.8 to 6.04 g/kg and met the WHO criteria of the product claim (± 25% g/kg). The insecticide un-impregnated control net has shown no detectable levels of alphacypermethrin. In this evaluation, HILNet® fulfilled the WHO laboratory (phase I) efficacy criteria based on the rates of knockdown up to 20 standard WHO washes.

Keywords Long-lasting insecticidal nets · Alphacypermethrin · Laboratory evaluation · Regeneration time · Wash resistance · *Anopheles culicifacies s.l*

Introduction

The two interventions for malaria control include artemisinin-based combination therapy (ACT) and vector control measures such as long-lasting insecticidal nets (LLINs), sustained by indoor residual spraying of insecticide (IRS) and intermittent preventive treatment in pregnancy (IPTp) (Corbel et al. 2010). In a 10-year review (2000–2010) of malaria control in Africa, the number of malaria cases decreased by 68% by use of LLINs, 10% by IRS, and 22% by ACT, i.e., mostly by LLIN (Bhatt et al. 2015). According to guidelines for malaria vector control (WHO 2019), "insecticide treated nets (ITNs) are those that repels, disables or kills mosquitoes that come into contact with the insecticide on the netting material."There are two types of ITNs conventionally treated nets (cITNs) and long-lasting insecticide treated nets (LLINs/LNs). The cITNs, despite being effective, are constrained in their use by low re-treatment rates and loss of bio-efficacy after washing (Lengeler 2004; Sampath et al. 1998). LLINs are synthetic fabric nets of polyethylene or polyester nets that are manufactured by insecticide incorporation or coating at the factory level and

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are expected to retain their biological activity for at least 20 standard washes under laboratory conditions and for 3 years of recommended use under field conditions (WHO 2013). Currently, LLIN refer to nets that are treated only with pyrethroid insecticide (https://www.who.int/teams/global-malaria-programme/prevention/vector-control/new-types-of-insecticide-treated-nets accessed on 15.09.2021). Presently, WHO recommended 22 prequalified LLINs of different fabrics and pyretrhroid insecticides manufactured by 13 companies that also included insecticide–insecticide/-synergist mixture nets (https://www.who.int/pq-vector-control accessed on 15.09.2021). In India, NVBDCP approved the use of polyethylene and polyester fabric nets impregnated with pyrethroid insecticides, alpha cypermethrin, or deltamethrin (https://nvbdcp.gov.in/WriteReadData/1982s/Guidelines-for-ITNS-LLINS.pdf accessed on 15.09.2021).

New brands of LLINs are tested in the laboratory (phase 1) followed by small-scale (phase II) and large-scale (phase III) evaluations in the field following the standard guidelines (WHO 2013). The nets that meet the WHO criterion of efficacy in both laboratory and field evaluations are recommended for use in the community. The evaluation of net is carried out in India in accordance with the standard protocols drafted by ICMR-NVBDCP (https://nvbdcp.gov.in/Doc/Revised-Common-Protocol-2014.pdf accessed on 28–12-2021).

India has reportedly six primary malaria vectors—*An. culicifacies*, *An. fluviatilis*, *An. stephensi*, *An. baimaii*, *An. minimus* and *An. sundaicus*; and four secondary malaria vectors—*An. annularis*, *An. philippinensis/nivepes*, *An. jeyporiensis*, and *An. varuna* (Raghavendra et al. 2011). Among these, *An. culicifacies*, the major vector species in rural and peri-urban areas contributes ~ 65% annual cases of malaria, *An. fluviatilis* ~ 15% in forested areas, and *An. stephensi*, ~ 12% of the malaria cases in urban areas in India (Sharma 1998). In India, LLINs are generally recommended to be used in rural settings where *An. culicifacies* transmits most of the malaria. The current paper present the results of the evaluation of an alphacypermethrin incorporated polyethylene LN (HILNET®) against the laboratory strain of insecticide-susceptible *An. culicifacies s.l.*. The evaluation of the net was made compliant to GLP norms. Regeneration time was determined i.e., the time required for regeneration of insecticide in the LLIN after 3 consecutive standard WHO washings by determining the bio-efficacy of nets against insecticide susceptible *An. culicifacies s.l.* in WHO cone bioassays. Wash resistance was determined against insecticide susceptible *An. culicifacies s.l.* at 0, 1, 3, 5, 10, 15, and 20 washes using WHO cone bioassays. Chemical content in net samples was determined at 0, 1, 3, 5, 10, 15, and 20 washes following CIPAC MT/454/LN/M/3.2 methods in a GLP certified lab.

Materials and methods

Mosquito net

The study was conducted on HILNET®-LN, 100 denier polyethylene net, white in color, 152 mesh size, 48.8 GSM, and bursting strength of 676 kPa incorporated with alpha-cypermethrin @ 6.6 g (a.i.)/kg (w/w) [provided by M/S HIL (India) Ltd, New Delhi].

Mosquito strain

An. culicifacies s.l. collected from Dehra, district Ghaziabad, Uttar Pradesh was established in the year 2011 at NIMR insectary following standard colonization procedures. The insecticide susceptibility was assessed periodically using standard WHO tube test method by using WHO kit and impregnated papers of prescribed discriminating dosages, DDT, 4.0%; malathion, 5.0%; deltamethrin, 0.05%; and alphacypermethrin, 0.05% (WHO 2016). *An. culicifacies s.l.* was completely susceptible to DDT, malathion, deltamethrin, and alphacypermethrin.

Net sampling

Four candidate LNs selected randomly from two different production batches (provided by the Manufacturer) were used. From each net, 14 pieces (25 cm × 25 cm) were sampled (Fig. 1). The tests were conducted on 52 net pieces as described in the flow chart (Fig. 2). Eight pieces (four unwashed and four washed) were used to estimate regeneration time and 24 net pieces were used to determine wash resistance [four pieces were tested after 1, 3, 5, 10, 15, and 20 washes (4 × 6 = 24 bioassays)]. Net pieces were wrapped individually in aluminum foil and stored at 30 °C until the conduct of bio-efficacy studies for both regeneration time and wash resistance studies using WHO cone bioassays. Twenty net pieces (five pieces HP1 to HP5 each from the four nets) (Fig. 2) were wrapped in aluminium foil and stored at 4 °C for chemical analysis to determine alphacypermethrin (w/w—gm (a.i.)/kg) in the net pieces and variability between the net pieces.

Washing procedure

Sampled net pieces (25 cm × 25 cm) cut from the nets (Fig. 1) were washed following standard WHO washing procedure (WHO 2013). The net peices were introduced individually in a 1 litre glass bottle (Duran®DWK Life Sciences GMBH, Germany) containing 0.5 L deionized water with 2 g/l soap. Recommended standard soap, Savon de Marseille (Marseille, France) was used for the washing.

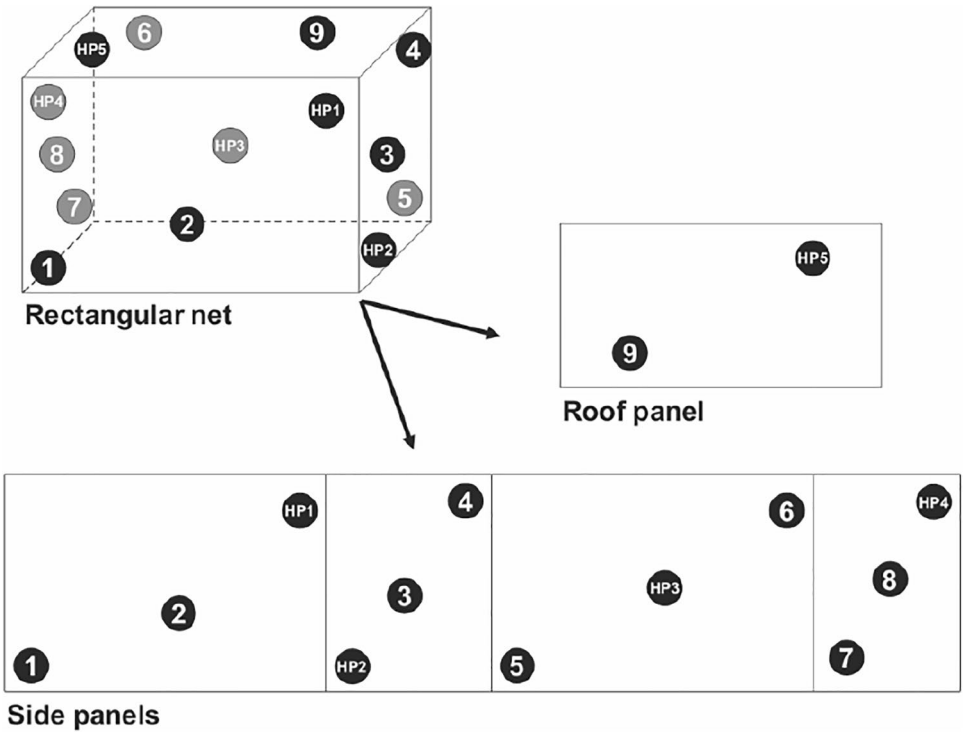

Fig. 1 Sampling scheme for 14 pieces of netting from each net, including positions HP1–HP5 for chemical assay (Source: WHO 2013)

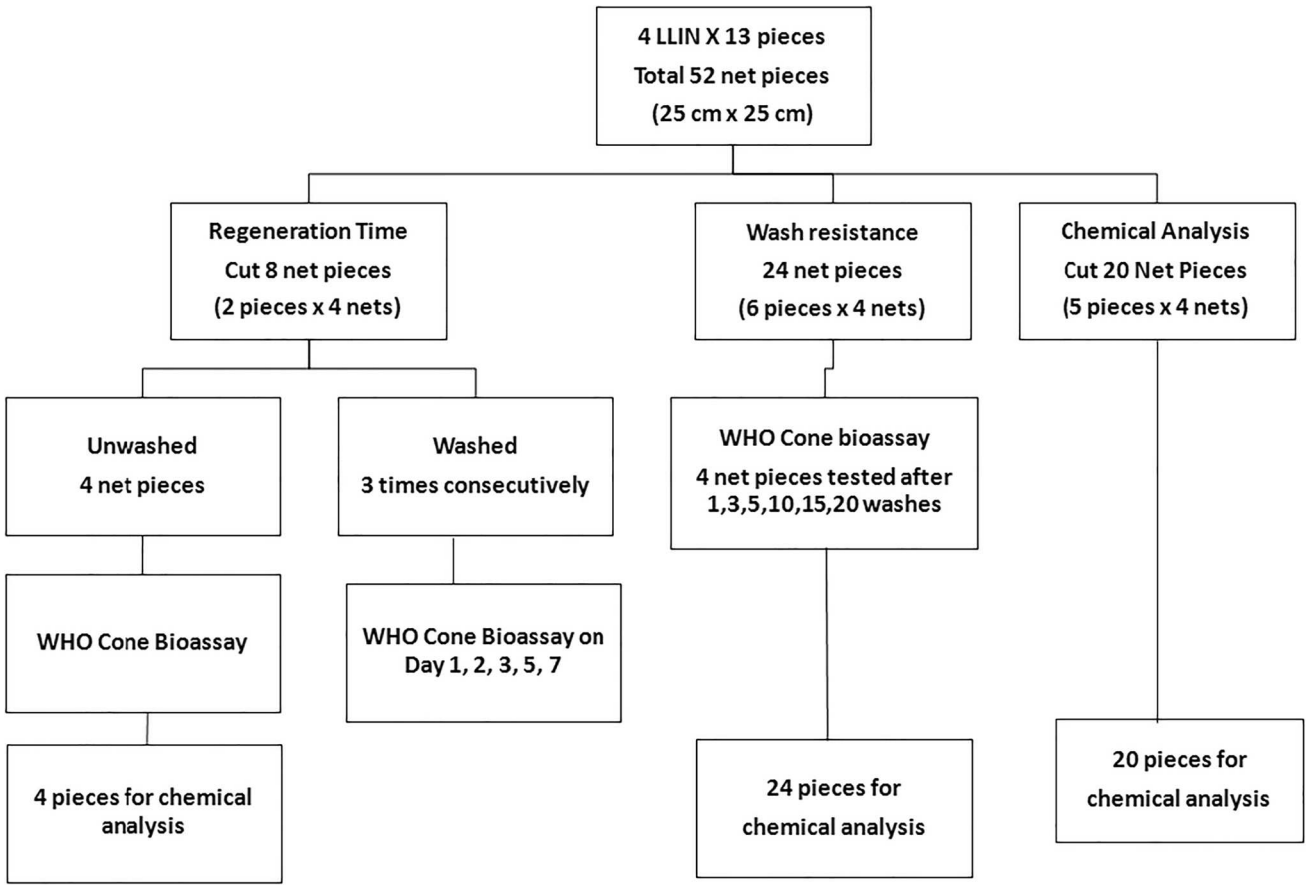

Fig. 2 Schematic flow of work plan

The bottles containing the net pieces were placed in shaker water bath (New Brunswick Scientific Co., Inc. USA) maintained at 30 °C and shaken for 10 min at 155 movements per minute. The soap washed net pieces were rinsed twice for 10 min in clean, deionized water under the same conditions in water bath, and dried at room temperature and stored at 30 °C between the washes. The net peices after the designated number of washes are stored at 30 °C until WHO cone bioassay to determine bio-efficacy.

WHO cone bioassay

Four WHO cones were fixed on a piece of netting mounted on an acrylic frame and secured with binder clips as described in guidelines (WHO 2013). Five non-blood-fed, 2–5-day-old alphacypermethrin susceptible female *An. culicifacies s.l.* mosquitoes were exposed to each piece of netting for 3 min. This procedure was repeated in replicates until a total of 50 mosquitoes were exposed to each piece. Simultaneously, 5–5 mosquitoes were exposed in 2 replicates on untreated net pieces as control. Knock-down rates of mosquitoes exposed to treated nets were recorded at 60 min after 3 min exposure and mortality was observed after 24 h holding period. The exposed mosquitoes were transferred to plastic bowls covered with nylon net fastened with rubber bands. The mosquitoes were provided access to 10% sucrose soaked cotton swabs placed on the net of the plastic bowls. The plastic bowls were kept in climatic chambers for 24 h at 27 ± 2ºC and 80% ± 10% RH. Percent mortality after 24 h holding period was calculated by scoring the alive, knock-down and dead mosquitoes based on the classification of WHO (2016). Mosquito is considered (a). alive if it can both stand upright and fly in a coordinated manner (b). moribund if it cannot stand (e.g. has one or two legs), cannot fly in a coordinated manner or takes off briefly but falls immediately are classified as knocked down dead if it is immobile and (c). dead if the mosquito cannot stand or show no sign of life (WHO 2016).

Regeneration time

The regeneration time is the time period required to regenerate insecticide in an LLIN after three consecutive standard washes (washing procedure described above) in a single day to deplete the insecticide on the net surface. The washed net pieces were wrapped in labeled aluminium foil and stored at 30 °C until WHO cone bioassays. Cone bioassays were conducted by exposing susceptible *An. culicifacies s.l.* for 3 min in WHO cones on day 1, 2, 3, 5 and 7. Knockdown rate at 60 min and mortality rate after 24 h holding at 27 ± 2ºC and 80% ± 10% relative humidity were recorded. Efficacy curves were drawn for knock down and mortality rates *vs* days. The time required (in days) to reach a plateau was considered as regeneration time (WHO 2013).

Wash resistance

The resistance of an LLIN to retain bio-efficacy up to 20 repeated washes (washing procedure described above) at intervals corresponding to the regeneration time in days is determined by exposing the susceptible *An. culicifacies s.l.* in WHO cone bioassays. Initially the bio-efficacy of unwashed nets was assessed. One piece of net was selected randomly from each of four nets at each wash. Each of the 4 pieces was washed once (1), 3, 5, 10, 15 and 20 times. The net pieces after designated washes were wrapped in aluminium foil and stored at 30 °C until WHO cone bioassays were performed. Thus, for each net 200 mosquitoes are tested for bio-efficacy assessment (4 pieces' × 10 cone tests × 5 mosquitoes = 200 mosquitoes). Knock-down rates were recorded at 60 min after 3 min exposure and mortality after a 24-h holding period with access to sugar solution at 27 ± 2 °C and 80% ± 10%RH. Mosquitoes exposed to untreated net pieces were considered as control replicates.

Chemical analysis

Netting samples derived after different washes were wrapped individually in aluminum foil and stored at 4 °C until analyses for the insecticide content in the net and to calculate their wash-resistance index (WHO 2013). The net samples were analyzed to estimate the chemical content (g a.i./kg) and variation between- and within-net following standard CIPAC MT/454/LN/M/3.2 methods in a GLP certified laboratory. The wash resistance index is defined as the proportion of the content of the active ingredient on/in the LN after washing compared to the insecticide content before washing. A total of 52 net pieces were analyzed for chemical content, 48 pieces as described in Figs. 1 and 4 net pieces from 4 insecticide untreated control nets.

Efficacy criteria for laboratory (phase I) studies

Nets washed at least 20 times that meet the criteria of WHO cone bioassays (≥ 95% knock-down or ≥ 80% mortality) or of the tunnel test (≥ 80% mortality or ≥ 90% blood-feeding inhibition) for undergoing field testing and evaluation (WHO 2013).

Results

Regeneration time

The average % knockdown in the net pieces from the four nets was in the range of 95–100% complying to the WHO criteria for knockdown ≥ 95% (WHO 2013). The average % mortalities of the 4 net pieces were in the range of

37.5–86.8% and were not in compliance to the WHO criteria of ≥ 80% except for the unwashed net. The time required (in days) to reach a plateau was considered as regeneration time (Table 1, Fig. 3).

As per the WHO criteria, the net has complied to the criteria of knockdown (≥ 95%), and there were no significant differences between mortalities at days 2, 3, or 5 (Pairwise comparison, Chi^2 $p > 0.05$). Based on the 7 days' data and the plateau in % KD for 2, 3, and 5 days, the observed regeneration time for HILNET® was considered as 2 days and being the minimum period.

Wash resistance

The nets were washed at intervals of 2 days, the determined regeneration time of the net. The % knockdown and % mortality was 0 (zero) for the untreated control net and the average % knockdown for the test net was in the range of 97–100%. The % knockdown recorded 100% up to 15 washes that decreased to 97% at 20th wash. The observed average % mortality for test net was observed in the range of 92–18% for 0 wash to 20 washes, while it was 92% for unwashed net, percent mortality successively decreased in each wash from 78% in 1st wash to 18% in the 20th wash (Fig. 4 and Table 2). The % mortality did not comply to the WHO criteria of ≥ 80% for recommendation of net similar to the observations in the studies on the determination of regeneration time. The net has complied with the WHO criteria of knockdown (≥ 95%) and met the WHO criterion of evaluation. Hence, the nets were not subjected to further evaluation using tunnel test.

Chemical analysis

The insecticide content of each net sample was analyzed to estimate between- and within-net variation, following standard CIPAC MT/454/LN/M/3.2 method in a GLP-certified

Table 1 Average % knockdown (% KD) and % mortality (%M) in cone bioassays on different days for determination of regeneration time

Day	Net 1 (side)		Net 2 (side)		Net 3 (roof)		Net 4 (side)		Average % KD	Average % mortality
	%KD	%M	%KD	%M	%KD	%M	%KD	%M		
Day 0	100	80.1	100	100	100	69.2	100	98	100.0	86.8
Day 1	94	46	100	48	100	50	86	37	95.0	45.3
Day 2	100	34	100	56	100	48	100	12	100.0	37.5
Day 3	92	30	100	50	100	52	100	24	98.0	39.0
Day 5	100	33.3	100	36	100	47.1	100	35.3	100.0	37.9
Day 7	100	44	100	50	100	82	100	27.4	100.0	50.9

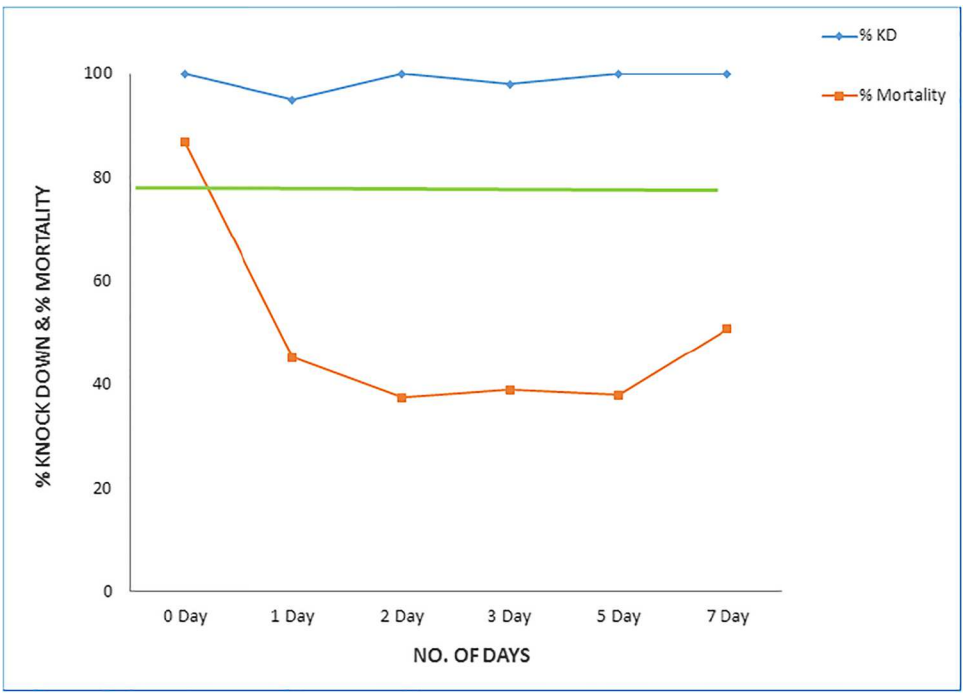

Fig. 3 Graph showing % KnockDown (%KD) and % Mortality in cone bioassays to determine the regeneration time of HILNet

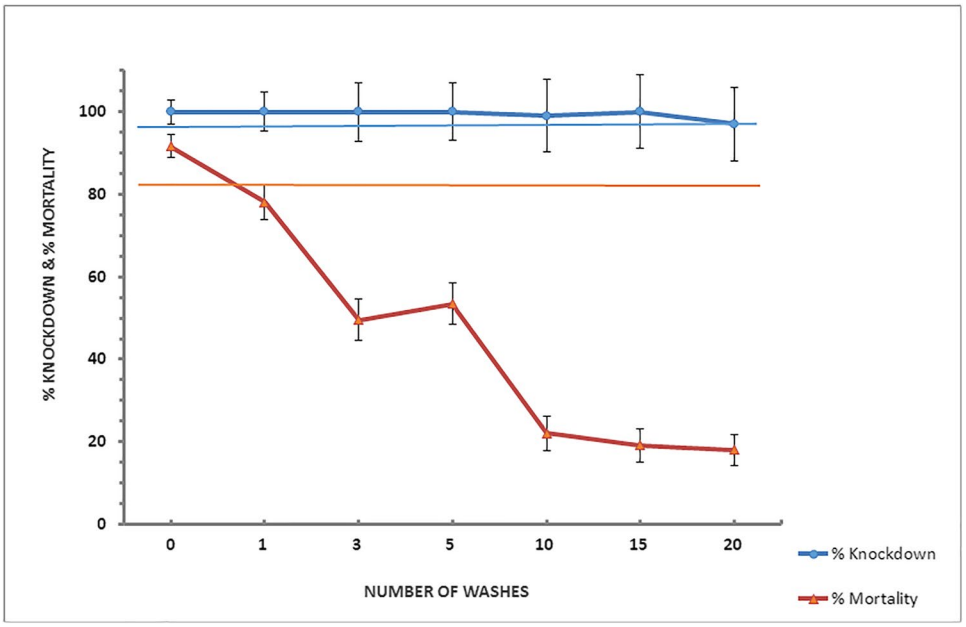

Fig. 4 Graph showing % KnockDown and % Mortality in wash resistance studies

lab. The wash-resistance index of the LLIN was calculated based on the decrease in insecticide content after repeated washings. The mean active ingredient (AI) content from 0 to 20 washes was in the range of 5.8 to 6.04 (g/kg) meeting the WHO criteria of the AI content ± 25% of the claim. However, after 5 washes, the mean content was 4.88 g/kg which is slightly below the lower rate of 4.95 g/kg (manufacturer claim is 6.6 g/kg alphacypermethrin), which later increased at wash 10 (Table 2) and beyond agreeing to the WHO criteria. The wash resistance index was above 95% in the 20 washes as per the criteria. The untreated control net has shown no detectable levels of alphacypermethrin A.I.

Discussion

The LLIN evaluated in this study is a polyethylene fabric and alphacypermethrin net (HILNet® LN). The specifications of HILNET®-LN were 100 denier polyethylene net white in color, a 152-mesh size, 48.8 GSM, and bursting strength of 676 kPa incorporated with alphacypermethrin @ 6.6 g/kg(w/w). There are reported 4 (four) WHO pre-qualified LLINs with polyethylene and alphacypermethrin namely, DuraNet® LN—5.8 g a.i /kg (WHO 2007), MAGNet™ LN -5.8 g a.i/kg (WHO 2011), Royal Sentry® LN—5.8 g a.i /kg (WHO 2011), and MiraNet LN -4.5 g a.i /kg (WHO 2015) and have mesh size in the range of 18–20 holes/cm^2, denier 135–150, and bursting strength in the range of 400–470 kPa, while alphacypermethrin interceptor LN is of polyester fabric and manufactured by incorporation technology with 6.7 g a.i./kg, 24 holes/cm^2, 75 denier, and bursting strength of not less than 250 kPa (https://extranet.who.int/pqweb/vector-control-product/interceptor accessed on 29.11.2021).

The determination of regeneration time in LLIN is dependent on bioavailability of insecticide to the mosquitoes and generally show variation for different nets. The determined regeneration time alphacypermethrin LNs was 1 (one) day for DuraNet (WHO 2007), MAGNet (WHO 2011),

Table 2 Table showing % average knockdown, % average mortality and mean alphacypermethrin AI (g/kg) in the nets at 0,1,3,5,10,15 and 20 washes

Number of washes	Control net		Test net		
	% Knockdown	% Mortality	Average % Knockdown ± SE	Average % Mortality ± SE	Mean AI(g/kg) ± SE
0	0	0	100 ± 3.22	91.68 ± 3.08	5.8 ± 0.186
1	0	0	100 ± 5.23	78.11 ± 4.62	5.57 ± 0.140
3	0	0	100 ± 7.98	49.52 ± 5.62	5.88 ± 0.639
5	0	0	100 ± 7.76	53.48 ± 5.68	4.88 ± 0.143
10	0	0	99 ± 9.85	22.09 ± 4.65	6.04 ± 0.144
15	0	0	100 ± 10.03	19.10 ± 4.38	5.10 ± 0.308
20	0	0	97.1 ± 9.96	18 ± 4.29	5.77 ± 0.190

and Royal Sentry net (WHO 2011), while it was 2 days for MiraNet (WHO 2015). In this evaluation study, based on the observed knock down rates, the regeneration time of HILNet® was determined as 2 (two) days.

In the wash resistance studies, all the sampled 4 HILNet® nets taken from 2 different batches have shown good efficacy, with recorded knockdown rate of 100% up to 15 washes and 97% at 20th wash in WHO cone bioassays and complied to the criterion of WHO for the recommendation of the efficacy of LLIN. In studies on alphacypermethrin LNs, Royal Sentry (WHO 2011), DuraNet (WHO 2007), MiraNet (WHO 2015), and in MAGNet (WHO 2011), % KD was in the range of 98–100 while % mortality was found to differ. For Royal Sentry LN, % mortality was 79 until 20 washes and 87 at 25th wash (WHO 2011), for DuraNet LN, it was 98% until 5 washes, and dropped to 45% at 20th wash (WHO 2007) while in MiraNet, mortality recorded was 68% by 20th wash and in MAGNet it was 100 in first 10 washes and 97% at 20th wash (WHO 2011). Thus, Royal Sentry® and MAGNet™ LNs fulfilled the WHOPES phase I efficacy criteria based on the knockdown and mortality rates while DuraNet LN and MiraNet LN fulfilled this criterion based on knockdown rates alone. Similarly, the HILNet® in this study fulfilled the WHO phase I laboratory efficacy criteria based on the rates of knockdown up to 20 standard WHO washes.

Conclusion

The alphacypermethrin LN (HILNet®) in the present laboratory evaluation (phase 1) complied to the prescribed WHO criterion of knockdown rate of ≥ 95%. The net can be evaluated further for phase 2 and phase 3 evaluations.

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Author contribution VV and RK conducted the study. VV and DK analyzed the data and prepared the illusrations. VV, RK, and MR drafted the MS. All authors saw the draft and approved it.

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Data availability Not applicable.

Declarations

Ethics approval The approval for conducting the study was taken from Indian Council of Medical Research, New Delhi on 08th August, 2019 (File No:5/8– 7(224)/2016/ECD-II Dated 8th August, 2019). Institutional Animal Ethics Committee clearance was obtained for feeding mosquitoes on rabbit for colonization in Insectary.

Consent to participate Not applicable.

Consent for publication The permission to publish this manuscript was taken by Research Ethics Committee of ICMR-National Institute for Malaria Research, Delhi, India with approval number RIC-54/2021.

Conflict of interest The authors declare no competing interests.

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