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Source: Journal of the American Mosquito Control Association, 27(4) : 423-428

Published By: The American Mosquito Control Association

URL: https://doi.org/10.2987/11-6170.1

Journal of the American Mosquito Control Association, 27(4):423–428, 2011

WASH RESISTANCE AND EFFICACY OF OLYSET® NET AND PERMANET® 2.0 AGAINST *ANOPHELES STEPHENSI* IN INDIA

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ABSTRACT. Long-lasting insecticidal nets (LLIN) have been developed for wash resistance and long-lasting effects against mosquito vectors. In this study we evaluated 2 LLIN products, Olyset® net and Permanet® 2.0, washed for 0, 5, 10, 15, and 20 times, against *Anopheles stephensi*, an urban malaria vector in India. We assessed the wash resistance and efficacy of these nets in relation to bloodfeeding inhibition and percent mortality in cone and tunnel test bioassays. Both Olyset and Permanet showed >80% mortality of *An. stephensi* in cone bioassays after 20 washes. In tunnel tests there was no significant difference between Olyset and Permanet 2.0 in causing total mosquito mortality (immediate and delayed) up to 10 washes and bloodfeeding inhibition and entry rate up to 15 washes. After the 20th wash, Permanet 2.0 was significantly more effective than the Olyset net in causing total mosquito mortality, whereas Olyset net showed less bloodfeeding and entry of mosquitoes as compared to Permanet 2.0. There was a gradual decline in efficacy of both LLIN products as the number of washings increased. Cone bioassays indicated a strong wash resistance in both the LLIN products after 20 washes. However, the tunnel tests demonstrated a gradual decline in efficacy of both products with the number of washings.

KEY WORDS Long-lasting insecticidal nets, cone bioassays, tunnel tests, bloodfeeding inhibition, entry rate

INTRODUCTION

Vector control is a prime requisite for managing many emerging and reemerging mosquito-borne diseases such as dengue, malaria, and chikungunya. Many new innovations have the potential to reduce the level of burden of vector-borne diseases. Bed-nets are commonly used as a method of protection against mosquitoes. Several workers have studied the efficacy of nets treated with pyrethroids (Hougard et al. 2003, Vatandoost et al. 2006, Babaee et al. 2007, Pennetier et al. 2007). Long-lasting insecticidal nets (LLIN) have emerged as a potential tool to fight the morbidity and mortality related to vector-borne diseases. These nets are biologically active for about 3–5 years. At present there are only 3 LLIN products, Olyset® net (Sumitomo Chemicals, Tokyo, Japan), Permanet® 2.0 (Vestergaard Frandsen, Lausanne, Switzerland), and Yorkool® net (Tianjin Yorkool, Tianjin, China), with full recommendation from the World Health Organization Pesticide Evaluation Scheme for use in vector control, while some others have gotten only interim recommendations (WHO 2011). It is essential to study the impact of these nets under real conditions on target vector populations in order to predict their impact on transmission, particularly changes in bloodfeeding inhibition and mortality. The standard cone test has yielded highly variable results (Graham et al. 2005, Jayalakshmi 2006, Gunasekaran and Vaidyanathan 2008, Rafinejad et al. 2008, Sreehari et al. 2009). To avoid inconsistency in assays and limit the risk of underestimating efficacy of LLINs, a more comprehensive testing of LLINs includes the tunnel test in addition to the cone test (WHOPES 2005). In the present study, we evaluated the effect of washing of LLINs up to 20 washes on efficacy of these nets in relation to bloodfeeding inhibition and percent mortality against malaria vector *Anopheles stephensi* Liston using laboratory cone and tunnel bioassays.

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MATERIALS AND METHODS

Two LLINs, Olyset net (polyethylene net incorporated with 2% permethrin) and Permanet 2.0 (polyester net coated with 55 mg/m^2 deltamethrin), and laboratory-reared pyrethroid-susceptible *An. stephensi* were used in this study. To assess the wash resistance of LLINs washed for 0, 5, 10, 15, and 20 times, LLINs were tested against adult *An. stephensi* in cone and tunnel bioassays. For the assays, cut pieces of each net measuring 25 × 25 cm were washed with a commercial detergent, Surf Excel (Hindustan Uniliver Private Ltd., Mumbai, India). One teaspoon (~5 g) of the detergent was dissolved in 5 liters of water and net pieces were dipped in the detergent solution for 10 min. The pH of detergent solution was 9.0 to 9.5. Subsequently, the net pieces were thoroughly rinsed with tap water and dried under shade for 8 h. After drying, these nets were packed in a polyethylene bag and stored in a cupboard at room temperature until the next wash. All the nets were washed at 7-day intervals. In cone bioassays five 3-day- to 5-day-old test mosquitoes were exposed to netting materials for

3 min. After the 3-min exposure, mosquitoes were removed from cone using a suction tube and put in paper cups. The mosquitoes were provided with cotton soaked in 5% glucose solution and placed on the net covering the paper cup. Next, the paper cups were kept in an environmental chamber maintained at 27 ± 2°C and 80% RH. Knockdown was scored after 1 h and mortality after 24 h. For each wash, cone bioassays were performed in 4 replicates. Untreated nets were used as control.

Tunnel tests were carried out with slight modifications in the World Health Organization procedure. The tunnel (25 × 25 × 60 cm) was fabricated glass (Elissa and Curtis 1995) with a piece of LLIN mounted on a disposable cardboard frame placed across the tunnel 25 cm from one end. The surface area of netting accessible to mosquitoes was 20 × 20 cm with 9 holes, each 1 cm in diam; 1 hole was located at the center of the square with the other 8 holes equidistant and located at 5 cm from the border. Inside the shorter section of the tunnel, bait (a rabbit) was placed, which was unable to move but available to be bitten by the mosquitoes. At each end of the tunnel, a 30-cm-cube cage was fitted, covered with polyester netting. In the cage at the end of the longer section of the tunnel, unfed 96-h-old female mosquitoes were introduced and kept for bloodfeeding for 6 h in the dark room. To encourage bloodfeeding, we used 96-h-old mosquitoes rather than 48-h-old mosquitoes. The mosquitoes were free to fly in the tunnel and able to contact the LLIN netting and locate a hole to feed on the bait. After the blood meal, mosquitoes usually flew to rest at the end of the same section of the tunnel. After 6 h, the females were removed and counted separately from each section of the tunnel and the immediate acute mortality was recorded. Live females were put in plastic cups with 5% glucose solution and delayed mortality was recorded after 24 h. During exposure, cages were maintained in an environmental chamber at 27 ± 2°C and 80% RH under subdued light. Two tunnels were used simultaneously in the same climate chamber, one serving as a control. Three replicates of each wash were done along with the control tunnel. Three replicates were repeated on 3 consecutive days under the same conditions for each wash. Each net sample was used once and then discarded.

The reduction in bloodfeeding was assessed by comparing the proportion of bloodfed mosquitoes, whether dead or alive in tunnels with LLIN and untreated nets. The entry rate was measured as the proportion of mosquitoes that pass through the netting, by comparing LLIN and control net tunnels. Overall mortality was measured by combining both immediate and delayed (24 h) mortality of mosquitoes.

Table 1. Percent mortality (24 h) of *Anopheles stephensi* exposed to Olyset® and Permanet® 2.0 nets after different numbers of washes in cone bioassays.

No. washes	Olyset net	Permanet net	*P*-value
0 (fresh nets)	100 ± 0	100 ± 0	—
5	100 ± 0	100 ± 0	—
10	90 ± 0.57	95 ± 0.5	0.53696
15	85 ± 0.5	85 ± 0.5	—
20	85 ± 0.5	85 ± 0.5	—

Data analysis

For each treatment, the bloodfeeding inhibition rate (BFI) was calculated using the following formula: BFI = 100 − ([treatment feeding/control feeding] × 100). When control mortality exceeded 5%, treatment mortality was corrected (Abbott 1925). The *z*-test of proportions was used for comparing *An. stephensi* in experimental and control tunnels. Independent *t*-test was used to compare the mean values of entry rate, bloodfeeding, and total mortality between Olyset net and Permanet 2.0 using Epi info Open Source Epidemiologic Statistics for Public Health, version 2.3.1 (Centers for Disease Control and Prevention, Atlanta, GA). Regression graphs were prepared using the Free Statistics Software, Office for Research Development and Education, version 1.1.23-r7 (Wessa 2011), for calculating the change in total mortality, feeding rates, and entry rates after different washings in both the nets.

RESULTS

In cone bioassays, both the LLINs resulted in 85–100% mortality of the test mosquitoes after 20 washes, which exhibited their wash resistance (Table 1).

For the total mortality in tunnel bioassays, the fresh Olyset net showed 100% total mortality of *An. stephensi* (z-value = 13.72, $P < 0.05$), but as the washing progressed there was a significant decline in the total mortality values from 95% (5-times-washed nets, z-value = 12.74, $P < 0.05$) to 78.3% (z-value = 12.05, $P < 0.05$) in the case of the 20-times-washed nets (Table 2). Similarly, the Permanet 2.0 also showed 100% total mortality with fresh nets in comparison to control netting, but the total mortality values decreased from 94.3% on 5-times-washed nets, to 82% on 20-times-washed nets (z-value = 11.69, $P < 0.05$), indicating a lesser impact of washing on Permanet 2.0 than on Olyset net. The regression graphs clearly indicate that Olyset net lost more insecticide with increased washing ($Yt = 100.64 + -1.116Xt$) as compared to Permanet ($Yt = 99.52 + -0.906Xt$) (Fig. 1).

The entry rate of unfed mosquitoes toward the bait side was 0% in the case of fresh Olyset nets, increasing gradually on washed nets from 5 to 20

Table 2. Comparison between the efficacy of Olyset® net and Permanet® 2.0 against *Anopheles stephensi* after different numbers of washes as determined by tunnel tests. *P*-values shown indicate the level of difference between Olyset net and Permanet 2.0 as determined by the *t*-test.

No. washes	Olyset net	Permanet 2.0	*P*-value[1]
Total mortality of *An. stephensi* exposed to Olyset and Permanet 2.0 nets			
0 (fresh nets)	100 ± 0	100 ± 0	—
5	95 ± 1.1	94.3 ± 1.5	0.538
10	91.5 ± 1.5	91 ± 1	0.656
15	82.6 ± 1.1	85 ± 1	0.049*
20	78.3 ± 2	82 ± 1	0.045*
Entry rate of *An. stephensi* to Olyset and Permanet 2.0 nets			
0	0 ± 0	1.3 ± 1.1	0.110
5	5.33 ± 1.1	5.3 ± 1.1	0.975
10	9 ± 1.73	8.6 ± 1.5	0.777
15	21.3 ± 2.5	22.6 ± 1.1	0.456
20	28 ± 1	36 ± 2.0	0.003*
Feeding success rate of *An. stephensi* between Olyset and Permanet 2.0 nets			
0	0 ± 0	0 ± 0	—
5	0 ± 0	0 ± 0	—
10	1.33 ± 1	3 ± 1	0.110
15	5 ± 1	6.3 ± 1.5	0.279
20	8 ± 1	10.6 ± 1.1	0.03881*

[1] Asterisk indicates significant difference ($P < 0.05$).

washes. The entry rate was 5.33% on 5-times-washed nets, which increased to 28% on 20-times-washed nets, showing a significant difference in the entry rates of mosquitoes (Table 2).

In the case of Permanet 2.0, the entry rate of *An. stephensi* was 1.3% on fresh nets, which increased to 5.3% on 5-times-washed nets (z-value = 8.271, $P < 0.05$) and to 36% on 20-times-washed nets (z-value = 4.966, $P < 0.05$) (Fig. 1). Thus, there was a significant difference in the entry rates of the *An. stephensi* on fresh and 20-times-washed Permanet 2.0 ($P < 0.05$). When the entry rates of *An. stephensi* on 2 LLINs were compared, there was no significant difference observed up to 15 washes, but after the 20th wash, Olyset net was found significantly more superior to Permanet 2.0. With every wash there was 1.4% increase in the entry rate of mosquitoes in Olyset net as compared to 1.73% increase in Permanet net after each wash (Fig. 2).

The feeding success rate of mosquitoes in the case of fresh Olyset nets was 0% as compared to 73.3% in control netting (z-value = 10.57, $P <$ 0.05). As the number of washes progressed, the feeding rate was also increased from 0% on fresh net to 8% on 20-times-washed nets (z-value = 9.47, $P < 0.05$) (Table 2). In case of Permanet 2.0, the percentage of bloodfeeding was 0% on fresh nets but as the number of washes increased, the percentage of feeding increased to 10.6% on 20-times-washed nets. Thus, the feeding success was less in the case of Olyset net as compared to Permanet 2.0 ($P < 0.05$). With each wash, the feeding rate increased by 0.42% in Olyset net as compared to 0.55% in Permanet 2.0 net. The percentage of bloodfeeding inhibition ranged from 100% (fresh net) to 89.5% in case of 20-times-washed nets in Olyset nets. In Permanet 2.0 the percentage of bloodfeeding inhibition ranged from 100% (fresh net) to 85.9% (20-times-washed nets) (Fig. 3).There was also significant difference observed in both the nets in bloodfeeding success between the washes (5, 10, 15, 20). Hence, Olyset net was found to be more effective as compared to Permanet 2.0 in preventing bloodfeeding.

DISCUSSION

The use of LLINs is a breakthrough in malaria vector control by preventing biting and killing mosquitoes attracted to humans. Long-lasting insecticidal nets have been shown to have a biological shelf life and wash resistance of at least 20 times and durability of about 3–5 years (Kulkarni 2006). It is important to consider behavioral aspect of the targeted vector, in order to sustain any control effort. If regular monitoring was not done, then these efforts in the field of vector control might get wasted. There is, therefore, a need for vigilance in the evaluation and monitoring of new LLIN products as they become widely available and acceptable as malaria vector control tools. These nets have been promoted for use in reducing human–vector contact for a long time. Guidelines on standardized procedures for washing and drying of LLINs are available for the purpose of comparison of similar products between different laboratories (WHOPES 2005). However, the information available on the effect of washing of current brands of LLINs is limited, especially when assessed under real conditions like tunnel tests where bait is provided for attracting the mosquitoes, and washed LLIN was used as a barrier against the vector species. In the present study, the cone bioassays indicate wash resistance of these nets even after 20 washes. Similar observations were made in an earlier study on hand-washed nets (Sreehari et al. 2009). However, our tunnel assay suggests that the cone assay underestimates the loss of protection from biting. Our results indicated a significant difference after 15 washes in the total mortality between washed Olyset net and Permanet 2.0 in tunnel test bioassays. There was decline in percent mortality in cone bioassays after 20 washes. It indicates the effect of washing on the efficacy of nets, which we also found in tunnel bioassays. It may be due to that one is in confined chamber, whereas the other takes advantage of the natural host-seeking behavior. The efficacy of Olyset net and Permanet

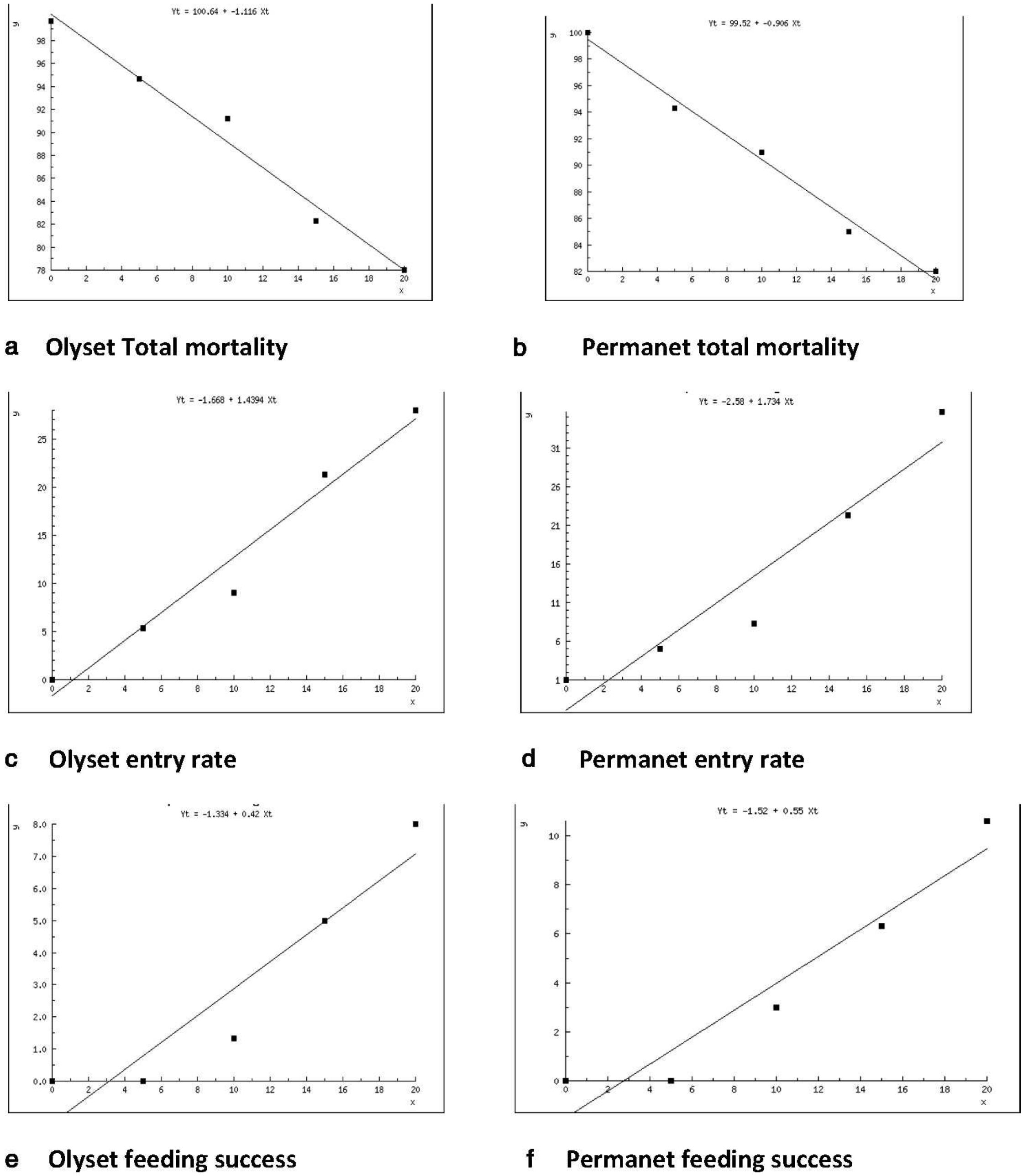

Fig. 1. Regression graphs of Olyset® net and Permanet® 2.0 between different numbers of washes and (a, b) total mortality, (c, d) entry rates, and (e, f) bloodfeeding rates (regression equations given in each graph). The *x*-axis represents the number of washes in each graph, and the *y*-axis shows the total mortality in A and b, entry rate in C and d, and feeding success in E and f.

2.0 against the pyrethroid-susceptible *An. stephensi* in terms of entry rates, feeding success, and total mortality showed a declining trend as the washing progressed (Fig. 1). In different tunnel assays, a 100% mortality on fresh Olyset nets and Permanet 2.0 was reduced to 96 ± 2 and 92 ± 2.7 after 20 washes, respectively (Rafinejad et al. 2008). The entry rate of *An. stephensi* in both the LLINs showed a significant difference between different numbers of washes with no significant difference in the entry rate of mosquitoes observed between the 2 nets. However, Olyset net when washed 20 times showed a lower entry rate as compared to Permanet 2.0 ($P = 0.003448$). No

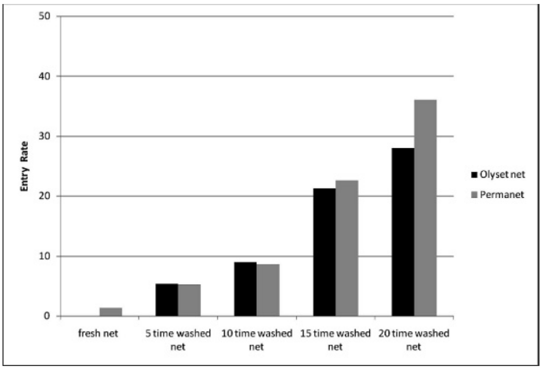

Fig. 2. Effect of washing of Olyset® net and Permanet® 2.0 on the entry rate of susceptible species *Anopheles stephensi*.

bloodfeeding was observed in unwashed nets and 5-times-washed nets in both LLINs. After 20 washings, although both the LLINs showed certain level of efficacy to reduce the levels of bloodfeeding, Olyset net showed a better impact in preventing the bloodfeeding than Permanet 2.0 ($P < 0.05$).

In a recent study, LLINs were found highly effective in preventing *An. gambiae* Giles from feeding before washing, but the feeding rate gradually increased with washing (Atieli et al. 2010). In our study, the effect of washing was not immediately evident. Up to 15 washes, there was no significant difference in feeding success rate or entry rate of mosquitoes between the Olyset net and Permanet 2.0. Overall, the feeding rate remained low and mortality remained high. *Anopheles stephensi* showed only 8% feeding and 79.6% mortality for Olyset nets and 10.6% feeding and 82% mortality for Permanet 2.0 after 20 washes.

These results indicate that permethrin possesses more repellency to the mosquitoes, as even after 20 washes it still showed efficacy in preventing mosquitoes from entering and feeding upon the bait. In an earlier study, permethrin-incorporated mosquito net showed stronger excito-repellency than most other pyrethroids (Hougard et al. 2003). This effect interferes with mosquito behavior during bioassays. Further, mosquitoes exposed to treated nettings can eventually be killed and/or repelled based on the residual concentration of permethrin at the surface of fibers and the test method used (RBM 2011).

Bioefficacy of LLINs depends upon various factors, and washing is one of the main factors in determining this parameter. Results have indicated that washing played an important role in the dilution of the efficacy of the LLINs, which further affects behavioral responses of vector mosquitoes in terms of bloodfeeding and entry towards the bait side. Washing removes the amount of insecticide from the fibers in both

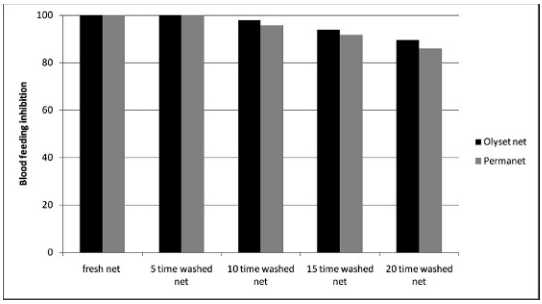

Fig. 3. Effect of washing of Olyset® net and Permanet® 2.0 on the bloodfeeding inhibition of susceptible species *Anopheles stephensi*.

the LLINs, as decreased rate of total mortality, bloodfeeding, and entry rate were observed in both cases. More studies are needed to understand the regeneration time of nets and how the coated and incorporated technologies affect the bioefficacy of LLIN after repeated washes.

ACKNOWLEDGMENTS

This study was undertaken with the support of a research grant provided by Indira Gandhi National Open University under the research and teaching assistantship scheme to the first author. The authors thankfully acknowledge the technical support provided by the staff of the National Institute of Malaria Research in carrying out this study. Research grants and the supply of Olyset net by M/S Sumitomo Chemicals India Pvt. Ltd. and PermaNet 2.0 by M/s. Vestergaard Frandsen India Pvt. Ltd. are also thankfully acknowledged.

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