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Wash resistance and efficacy of three long-lasting insecticidal nets assessed from bioassays on *Anopheles culicifacies* and *Anopheles stephensi*

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Summary

OBJECTIVE To test the wash resistance and efficacy of long-lasting insecticidal nets (LLINs), namely Olyset® Net and PermaNet® 2.0; and a long-lasting treatment kit, K-O Tab®1-2-3, on *Anopheles culicifacies* and *An. stephensi*, major malaria vectors in India, by bioassays. Conventionally treated deltamethrin net (CTDN with K-O Tab) was used for comparison.

METHOD Mortality and median time for knockdown (MTKD) of mosquitoes were determined using contact bioassays and ball frame bioassays respectively. Hand washing and machine washing were used.

RESULTS LLINs showed good bio-efficacy against *An. culicifacies* and *An. stephensi*. The mortality of mosquitoes remained >80% after up to 20 hand washes and up to 15 machine washes on all LLINs tested. No significant differences were observed in mortalities between the *An. culicifacies* and *An. stephensi* in cone bioassays ($P > 0.05$). MTKD increased progressively with successive washes and there was a significant difference in median time for knockdown of test mosquitoes and between hand-washed and machine-washed nets ($P < 0.05$).

CONCLUSION LLINs are more efficacious and last longer when washed by hand than by machine.

keywords long-lasting insecticidal nets, *Anopheles culicifacies, Anopheles stephensi,* mortality, median time for knockdown, wash resistance

Introduction

Long-lasting insecticidal nets (LLINs) are presently the most technologically advanced form and sustainable intervention for the vector control with proposed efficacy of 4–5 years that could facilitate scale-up for malaria prevention. A few brands of LLINs are already in use in some countries. WHO gives a full recommendation of Olyset® Net and an interim recommendation of PermaNet® 2.0, Duranet®, Net Protect® and Interceptor®. K-O Tab 1-2-3® and ICON® MAXX (long-lasting insecticide treatment kits) were also approved (WHO 2007).

Olyset net is treated at the factory with permethrin 2% incorporated into the yarns of the netting and the insecticide diffuses constantly over time to the surface of the yarn. The bioavailability of the insecticide is sufficient to kill mosquitoes. Efficacy trials of Olyset® Nets in Cambodia, Viet Nam, Tanzania, Solomon Islands, Malaysia, Senegal, Côte d'Ivoire and India reported encouraging results (Itoh & Okuno 1996; Nguyen *et al.* 1996; Vythilingam *et al.* 1996; Ikeshoji & Bakotee 1997; Faye *et al.* 1998; Doannio *et al.* 1999; Henry *et al.* 1999; N' Guessan *et al.* 2001; WHO 2001; Muller *et al.* 2002; Tami *et al.* 2004; Ansari *et al.* 2006; Jeyalaksmi *et al.* 2006; Maxwell *et al.* 2006; Sharma *et al.* 2006; Sreehari *et al.* 2007b).

PermaNet 2.0 coated with deltamethrin at the factory were evaluated for bio-efficacy in Pakistan, Tanzania, India, Uganda, and a few other countries and reported to perform well even after repeated washing (Kroeger *et al.* 2004; WHO 2004; Gimnig *et al.* 2005; Graham *et al.* 2005; Lindblade *et al.* 2005; Sreehari *et al.* 2007a; Gunasekaran & Vaidyanathan 2008).

Yates *et al.* (2005) carried out extensive studies on K-O Tab 1-2-3 treated nets in comparison to conventional K-O Tab and PermaNet and reported their wash resistance and efficacy even after 30 washes.

In India, malaria control is mainly focused on indoor residual spraying but insecticide treated nets (ITN) and LLINs are now also being promoted for the control of malaria vectors. Control of *An. culicifacies* seems the most promising approach as it transmits 60–70% of all malaria cases in India (Sharma 1998). We assessed the wash

resistance of Olyset and PermaNet 2.0 LLINs, K-O Tab 1-2-3 treated nets, and a conventionally treated deltamethrin net (CTDN with K-O Tab) by washing the nets with a washing machine and by hand rubbing. Bioassay tests were conducted on *An. culicifacies* and on *An. stephensi* to assess their efficacy against mosquitoes.

Materials and methods

We used *Anopheles culicifacies* and *An. stephensi* colonised at National Institute of Malaria Research, Delhi, India. The nets tested were Olyset Net (permethrin 2% incorporated into polyethylene net @ 1 g/m^2), PermaNet 2.0 (deltamethrin coated on polyester net @ 55 mg/m^2), nets treated with K-O-Tab 1-2-3 (a long-lasting treatment kit with deltamethrin @ 25 mg/m^2+ binder) and conventional deltamethrin nets (CTDN) treated with K-O Tab tablets (deltamethrin @ 25 mg/m^2). For impregnation with KO- Tabs, white untreated nets of 100-denier, 40 g/m^2 weight were used and impregnated as per the manufacturers' instructions.

To assess wash resistance of the LLINs, two types of washing procedures were followed. For each type of net, four replicates were used. One piece from each net measuring 1 m^2 was cut from four nets and washed. After drying the nets were packed in a polythene bag and kept in dark and cool place at ambient temperature in a cupboard until the next wash. All nets were washed with 'Surf Excel' (M/s. Hindustan Lever India Pvt. Ltd, Mumbai, India).

For handwashing, 10 g of detergent powder were dissolved in 10 l of water. The pH of detergent mixed water was 9.0–9.5. The batches of nets were soaked in the detergent water for 30 min, then thoroughly rubbed for 10 min, rinsed thrice in plain water and dried in the shade for 8 h. The initial water temperature was 29 ± 1°C. The temperature was recorded at 09:00, 13:00 and 17:00 hours.

For washing with a semi-automatic washing machine, only one type of net was washed at a time. The tub was filled with 35 l of tap water at 29 ± 1 °C and 20 g detergent powder were added and whirled thoroughly. Nets were soaked in the tub for 30 min, then whirled for 6 min. After washing, the detergent water was drained completely and the nets were rinsed for 3 min with fresh tap water. After complete draining, the nets were spun at 500 rpm for one minute and dried in shade for 8 h. To test the regeneration of permethrin on Olyset Nets, some nets washed 20 × by both methods were dried in sunlight and compared to those dried in shade.

All nets were washed at 7-day intervals. Bioassays on these nets were conducted on day 1, the day after the 5th, 10th, 15th and 20th wash against all the LLINs and in case of CTDN, on day 1 after impregnation and after washes 1, 3, 5, 7 and 10. All nets were washed 20 times.

Cone and ball frame bioassay tests were performed to test the efficacy of nets in producing mortality and knockdown. Three-minute cone bioassays were conducted on day 1 and ball frame bioassays on day 2 after the wash. Bioassay tests were performed as per WHO standard procedures (WHO 1998). After three minutes' exposure, mosquitoes were kept for recovery in bowls with access to 10% glucose soaked cotton for 24 h and mortality was scored. For each species four replicate nets were used. Untreated nets were taken as control. Data were pooled and percent-corrected mortality was calculated and corrected when the mortality in control replicates was >5 and <20% using Abbott's formula (Abbott 1925).

Ball frame bioassays were conducted according to WHO (1998) and the median time to knockdown of each mosquito species exposed to different LLINs was recorded. Eleven mosquitoes were introduced into the ball frame wrapped with test LLIN and the time of complete knockdown of I, VI and XI mosquito was recorded. The time taken for the knockdown of median mosquito (VI) was taken as the median knockdown time.

Statistical analysis was performed using Fisher's exact test, Student's *t*-test and ANOVA. Comparisons were made in between number of washes, species, LLINs and types of washes.

Results

Per cent mortalities and MTKD of *An. culicifacies* exposed from Olyset nets, Permanets, K-O Tab 1-2-3 and CTDN unwashed or washed are given in Table 1 and that of *An. stephensi* in Table 2. Almost 100% mortality was recorded in both *An. culicifacies* and *An. stephensi* exposed from all unwashed LLINs and CTDN. Statistical analysis showed that there was no significant difference in the mortality of *An. culicifacies* or *An. stephensi* exposed from different types of LLINs washed by machine or hand. All hand washed LLINs produced mortality of >80% up to 20 washes in both *An. culicifacies* and *An. stephensi*. In case of CTDN, >80% mortality was reported up to three - washes in both species. In case of machine-washed nets, >80% mortality was reported up to 10–15 washes only in all the LLINs and up to one wash only on CTDN in both the species. Analysis of variance showed no significant differences in mortality of *An. culicifacies* and *An. stephensi* mosquitoes exposed from all LLINs, indicating equal effectiveness of the three brands of LLINs tested. When 20 × machine or hand-washed Olyset nets were exposed to sunlight for 5 days continuously in a polythene bag (8 h/day) and bio-assayed on the 6th day, the

Table 1 Per cent mortality and mean ± SD time (in seconds) of knockdown of the median mosquito (MTKD) of *Anopheles culicifacies* mosquitoes exposed from different types of nets

	Mortality*			MTKD†		
Number of washes	Machine wash	Hand wash	Machine *vs.* hand wash (*P*)	Machine wash	Hand wash	Machine *vs.* hand wash (*P*)
Olyset net						
Unwashed	100[a]	100[a]	–	311 ± 7[a]	311 ± 7[a]	
5×	100[a]	100[a]	1	332 ± 6[b]	314 ± 5[a]	0.002
10×	92.5[a]	100[a]	0.24	385 ± 11[c]	351 ± 6[b]	0.001
15×	78.9[b]	87.1[b]	0.35	416 ± 7[d]	403 ± 9[c]	0.47
20×	62.5[b]	82[b]	0.07	457 ± 9[e]	443 ± 8[d]	0.03
20 × + heated	72.5[b]	85[b]	0.27	381 ± 13[c]	352 ± 6[b]	0.006
Permanet						
Unwashed	100a	100[a]	–	390 ± 7[a]	390 ± 6[a]	–
5 ×	100a	100[a]	1	406 ± 8[b]	397 ± 2[a]	0.048
10 ×	94.8[ab]	100[a]	0.24	472 ± 10[c]	415 ± 5[b]	0.0001
15 ×	81b[c]	89.4[ab]	0.34	505 ± 11[d]	446 ± 11[c]	0.354
20 ×	67.5[c]	80[b]	0.3	580 ± 5[e]	506 ± 9[d]	0.354
K-O Tab 1-2-3						
Unwashed	100[a]	100[a]	–	393 ± 6[a]	393 ± 6[a]	–
5 ×	100[a]	100[a]	1	413 ± 7[b]	402 ± 2[a]	0.017
10 ×	90[a]	95[a]	0.67	475 ± 5[c]	432 ± 7[b]	<0.0001
15 ×	82.5[bc]	89.7[ab]	0.51	517 ± 6[d]	454 ± 9[c]	0.0014
20 ×	67.5[c]	82.5[b]	0.19	600 ± 22[e]	510 ± 5[d]	0.0014
CTDN (K-O Tab)						
Unwashed	100[a]	100[a]	–	411 ± 15[a]	411 ± 15[a]	–
1 ×	87.5[a]	100[a]	0.054	509 ± 11[b]	481 ± 13[b]	0.009
3 ×	60b	90a	0.003	917 ± 44[c]	678 ± 14[c]	0.0003
5 ×	30.7[c]	61.5[b]	0.011	1293 ± 107[d]	909 ± 59[d]	0.0019
7 ×	7.5[d]	20[c]	0.19	3428 ± 664[e]	1154 ± 44[e]	0.0019
10 ×	0[e]	2.5[d]	0.49	NKD	2254 ± 297[f]	–

*Four replicate nets were used and approximately 10 mosquitoes were tested against each net. Data were pooled for each type of net.
†Time of knockdown of the median mosquito from a sample of 11 mosquitoes exposed.
Data in the same column for a particular net sharing the same superscript letter do not differ significantly ($P > 0.05$) (in between the washes). ANOVA results indicated no significant differences in mortality and MTKD among LLINs in respective washes. NKD, no knockdown.

mortality of both species rose compared to the respective nets that were dried in shade, indicating regeneration of permethrin on Olyset net after exposure to sunlight.

The time of knockdown of median mosquito differed significantly between machine washed and hand washed Olyset nets, K-O Tab 1-2-3 nets, CTDN and in few washes in case of Permanets. Analysis of variance showed no significant differences in MTKD of *An. culicifacies* among LLINs in both type of washes ($P > 0.05$). There was a significant difference in MTKD of *An. culicifacies* and *An. stephensi* ($P < 0.05$) in both types of washed nets. The time of knockdown of median mosquito increased progressively after progressive increase in number of washes in both the species. Regeneration of permethrin on Olyset net was observed in 20 × machine and hand-washed nets that were exposed to sunlight (35–43°C) for 5 days continuously and tested on sixth day showed less MTKD than that of 20 × machine or hand washed nets dried in shade. The MTKD of *An. stephensi* was higher than that of *An. culicifacies* ($P < 0.05$). There was a statistically significant difference in MTKD of test mosquitoes when compared in between machine-washed and hand-washed nets and also in between the number of washes ($P < 0.05$). Analysis of variance between LLINs showed no significant difference in MTKD in respective washes. In case of CTDN, after seven machine washes and 10 hand washes nets did not show any impact on mosquitoes as the MTKD was more than 2000 s. There was no knockdown of all mosquitoes in 1 h exposed to 10 times machine washed nets. There was no knockdown up to 1 h of *An. stephensi* exposed to 7× hand-washed net. The MTKD of all test mosquitoes on K-O Tab treated net were much higher than those of all the LLINs. The results indicate poor wash resistance of CTDN.

Table 2 Per cent mortality and mean ± sd time (in seconds) of knockdown of the median mosquito (MTKD) of *Anopheles stephensi* mosquitoes exposed from different nets

	Mortality*			MTKD†		
Number of washes	Machine wash	Hand wash	Machine *vs.* hand wash (*P*)	Machine wash	Hand wash	Machine *vs.* hand wash (*P*)
Olyset net						
Unwashed	100[a]	100[a]	–	349 ± 7[a]	349 ± 7[a]	
5×	100[a]	100[a]	1	395 ± 10[b]	363 ± 4[b]	0.0026
10×	86.1[b]	92.5[ab]	0.46	436 ± 9[c]	405 ± 5[c]	0.0013
15×	72.9[bc]	82.9[b]	0.41	467 ± 8[d]	444 ± 6[d]	0.0027
20×	62.5[c]	80[b]	0.13	541 ± 11[e]	502 ± 13[e]	0.0018
20 × + heated	72.5[bc]	82[b]	0.6	423 ± 7[c]	393 ± 5[c]	0.0002
Permanet						
Unwashed	100[a]	100[a]	–	446 ± 10[a]	446 ± 10[a]	–
5 ×	100[a]	100[a]	1	519 ± 18[b]	480 ± 13[b]	0.0075
10 ×	84.2[b]	92.5[ab]	0.3	557 ± 3[c]	538 ± 7[c]	0.018
15 ×	70b[c]	85[b]	0.17	621 ± 39[d]	599 ± 9[d]	0.179
20 ×	62.5[c]	82[b]	0.07	735 ± 27[e]	693 ± 8[e]	0.023
K-O Tab 1-2-3						
Unwashed	100[a]	100[a]	–	451 ± 8[a]	451 ± 8[a]	–
5 ×	90[ab]	100[a]	0.11	523 ± 16[b]	486 ± 12[b]	0.005
10 ×	85.3[b]	92.6[ab]	0.48	565 ± 9[c]	550 ± 6[c]	0.018
15 ×	83.3[bc]	87.5[b]	0.75	633 ± 32[d]	602 ± 10[d]	0.072
20 ×	70[c]	80.4[b]	0.31	757 ± 19[e]	697 ± 5[e]	0.0032
CTDN (K-O Tab)						
Unwashed	100[a]	100[a]	–	460 ± 20[a]	460 ± 20[a]	–
1 ×	87.5[b]	95[a]	0.154	582 ± 14[b]	507 ± 7[b]	<0.0001
3 ×	60[c]	82.5[b]	0.046	734 ± 16[c]	613 ± 10[c]	<0.0001
5 ×	30[d]	60[c]	0.012	1638 ± 31[d]	1295 ± 45[d]	<0.0001
7 ×	7.5[e]	30[d]	0.019	NKD	NKD	–
10 ×	0[e]	5[e]	0.049	NKD	NKD	–

Meaning of superscripts as in Table 1.

Discussion

The study revealed high efficacy of LLINs on mosquitoes tested. Mortality of both mosquito species exceeded 80% in nets washed up to 20 times by hand and up to 15 times by machine. There was no significant difference in mortalities between species indicating that the LLINs are effective against all the vector mosquitoes tested. Nguyen *et al.* (1996) reported that mosquitocidal activity of Olyset nets remained 100% up to 8 months on *Ae. aegypti*. In trials in Tanzania 3-min exposure induced 70–80% mortality in *An. gambiae* after six months and after 12 months. Times for 80% knockdown was about 5–6 min even after 12 months of use; after 24 h washed nets produced similar results to those of unwashed nets. Our results confirm those of other studies (Vythilingam *et al.* 1996; N' Guessan *et al.* 2001; Ansari *et al.* 2006; Jeyalaksmi *et al.* 2006; Maxwell *et al.* 2006; Sharma *et al.* 2006).

The high efficacy of Olyset nets even after repeated washings may be due to the regeneration of the insecticide. A major advantage of Olyset nets is that its biological efficacy is resumed by diffusion of the insecticide from the inside of yarn to the surface which is accelerated with the exposure to sunlight. This might be the reason for high efficacy of these nets even after repeated washings, which was also observed in the present study with washed nets dried under direct sunlight for 8 h (temperature 35–43°C). However, two recent studies by Lindblade *et al.* (2005) and Gimnig *et al.* (2005) reported poor performance of the Olyset nets in the laboratory against *An. gambiae*. The authors reported reduced mortality after repeated washings to <5% and restoration of biological activity of Olyset nets only after heating to 60 °C for 4 h. However, their method of bioassay was criticised by Maxwell *et al.* (2006). As indicated by the manufacturer, permethrin in Olyset nets regenerates when exposed to strong sunlight or deliberate heating over a period of time. In the present study too the regeneration of permethrin was clearly observed when the 20 × washed nets were dried in sunlight for 8 h a day for five days in a row. The mortality of all mosquito species

tested increased in cone bioassays and the MTKD was lower in nets dried in sunlight than the 20 × washed nets dried in shade.

The results of the present study revealed increased efficacy of Permanet in producing >80% mortality in all test mosquitoes up to 20 hand washes and up to 10 machine. After 20 machine washes the mortality was >60%. This confirms the findings of earlier studies (Kroeger *et al.* 2004; WHO 2004; Gimnig *et al.* 2005; Graham *et al.* 2005; Lindblade *et al.* 2005; Sreehari *et al.* 2007; Gunasekaran & Vaidyanathan 2008). Kroeger *et al.* (2004) reported > 80% mortality in *Anopheles* mosquitoes exposed to 23 times-washed PermeNet. Graham *et al.* (2005) reported >97% mortality in *Anopheles* mosquitoes exposed to 21 times-washed PermaNet and Gimnig *et al.* (2005) reported high mortality rates even after 20 washes. Gunasekaran and Vaidyanathan also reported >80% mortality in *An. stephensi* mosquitoes exposed to 26 times-washed Permanets.

The difference in MTKD of *An. stephensi* and *An. culicifacies* might be due to the differences in their behavioural adaptability. Progressive increase in MTKD was observed in both the species after successive washes. The results agree with the earlier findings of Kroeger *et al.* (2004) and Graham *et al.* (2005), who reported an increase in MTKD after washing. The cause for increase in MTKD in all the species might be due to the loss of insecticide after repeated washings. However, the amount of insecticide left over on the nets might have been sufficient to produce >80% mortality in these mosquitoes as evidenced in three minute cone bioassay tests. This shows the improved wash resistance of PermaNets.

The results of the present study showed wash resistance of K-O Tab 1-2-3 nets. The mortality of test mosquitoes remained >80% up to 20 hand washes and 15 machine washes. Even after 20 machine washes the mortality was >67% in all test mosquitoes. Similar results as that of Permanet were obtained for K-O Tab 1-2-3 nets also. Yates *et al.* (2005) carried out extensive studies on K-O Tab 1-2-3 treated nets in comparison to conventional K-O Tab and PermaNet and reported that mortality caused by K-O Tab treated net steadily decreased between 5 and 30 washes, and on K-O Tab 1-2-3 treated nets and PermaNet, almost 100% mortality was maintained even after 30 wash cycles. As per the manufacturer's instructions the duration of efficacy of K-O Tab 1-2-3 is about three years and the present laboratory results and that of Yates *et al.* (2005) suggest the same. CTDN (K-O tab treated nets) showed poor wash resistance. However, the effectiveness in actual field use and impact on malaria transmission are to be confirmed from long-term multicentric studies in different eco-epidemiological settings. In conclusion, hand wash is more appropriate to retain the efficacy of these LLINs than the machine wash.

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