

Comparison of Bio-efficacy of PermaNet 2.0 and PermaNet 3.0 Long Lasting Insecticide Nets against *Anopheles dirus*

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Long lasting insecticidal nets (LLINs) and insecticide treated bet nets have emerged as a potent tool globally in preventing morbidity and mortality due to malaria. The efficacy of the nets is depending on the washing frequency and duration of the nets used. For this purpose, we have tested the bio-efficacies of unwashed and washed PermaNet 2.0 and PermaNet 3.0 against laboratory-reared *Anopheles dirus* from Mudon Township, Mon State in laboratory from June 2013 to December 2014. Zero to 30 times washing (14 days interval) of LLINs nets and cone bio-assay test were done according to WHO 2005 standard method. Result found that *An. dirus* are susceptible to DDT4%, deltamethrin 0.05%, permethrin 0.75% and cyfluthrin 0.15%. Before and after repeated washing of both nets, knockdown and mortality rates of *An. dirus* were persisted 100% to unwashed and after 20 washes but the killing ability was gradually decline after 25 to 30 washes (90% to 55% for PermaNet 2.0 and 90% to 70% PermaNet 3.0). Laboratory cone bioassays confirmed the loss of biological activity of the PBO/deltamethrin-treated top panel after 25 to 30 times washing although killing ability of top panel was higher than the deltamethrin-treated side panel (95% to 75% for top and 85% to 65% for side panels).The bio-efficacy of PermaNet 3.0 showed no difference to PermaNet 2.0 when either unwashed or after 20 washes. Both products were highly protective 90% killing against main vector of *An. dirus* to 25 washes. In Myanmar, malaria is highly affected in deep forest areas, borderline areas and hard-to-reach areas where biological control methods are not possible. Effective alternative methods as deltamethrin treated PermaNet 2.0 and PermaNet 3.0 LLINs nets are required to distribute in these areas for controlling malaria transmission and major vectors of malaria.

Key words: PermaNet 2.0, PermaNet 3.0, Bio-efficacy, Knockdown, Mortality

INTRODUCTION

A long lasting insecticide treated net (LLIN) should retain its efficacy against vectors of malaria even after washing, meeting WHO requirements on knockdown and efficacy for 20 washes and more.¹ Insecticide treated bet nets have emerged as a potent tool globally in preventing morbidity and mortality due to mosquito-borne diseases, especially malaria². Although ITNs have been included in the national malaria control programs of many countries as vector

control option, low re-treatment rates of ITNs in most countries^{3, 4} are seriously affecting ITN programs.⁵ To meet this challenge, long lasting insecticidal nets (LLINs) have been developed incorporating synthetic pyrethroid in the net yarn at the manufacturing stage to ensure its bio-availability on the surface of the net even after multiple washing.⁶ The net must

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retain its effective biological activity without re-treatment for 20 washes and more WHO standard washes under laboratory conditions and three years of recommended use under field condition.⁷ Three LLINs have been fully recommended by the WHO and are now commercially available as PermaNet 2.0, Olyset and Yorkool LLIN.⁸

And additional six LLINs have an interim recommendation from WHOPES for the prevention of malaria and reduction of man-vector contact: Dawa Plus 2.0, PermaNet 2.5, permaNet 3.0, Netprotect, Duranet and Interceptor. PermaNet 3.0 is a new generation of long-lasting combination net developed by Vestergaard Frandsen, Denmark that incorporates the pyrethroid deltamethrin. It is an insecticide synergist-combination net, designed for use in areas where pyrethroid-resistant malaria vectors are prevalent. The product incorporates the synergist piperonyl butoxide (PBO) with a pyrethroid insecticide (deltamethrin) in the roof section. PBO is generally used in commercial aerosols for potentiating pyrethroid activity against P450s and esterases in flying or domestic insect pests.⁹

A synergist is a chemical that enhances the effect of the primary insecticide used; it increases the exposure of an insect to the insecticide as through the dose has been increased.¹⁰ The main way that PBO enhances the effects of deltamethrin is by inhibiting the metabolic enzyme defence system within the insect.¹¹

In Myanmar, malaria is endemic in many parts of the country and it is a priority public health problem. Nowadays, WHO recommended LLINs as PermaNet 2.0, Olyset, PermaNet 3.0 and other brands of LLINs made from other countries are distributed in malaria-prone areas and hard-to-reach areas by vector-borne diseases control programme (VBDC) MOH, Government societies, NGOs and INGOs in different parts of the country. Malaria is transmitted by *Anopheles* mosquitoes. Main vectors are *An. dirus* and

An. minimus which are highly harmful to man in malaria endemic areas. LLINs and ITNs lead to a reduction of human-vector contact and diminish mosquito population and also provide a physical barrier with high coverage levels that benefit the whole community. However, bio-efficacy of long-term insecticidal action of LLINs depends on the exposure of climate, washing frequencies and duration of use during the recommended period. Moreover, there is a lack of information about the impact of repeated washings and duration of use on insecticidal action of LLINs against *Anopheles* mosquitoes in Myanmar. Therefore, this study evaluated the bio-efficacy of PermaNet 2.0 and newly introduced market available PermaNet 3.0 long lasting insecticidal-treated net in relation to washing frequencies of nets against *Anopheles dirus* mosquitoes under laboratory condition to provide information gap concerning on retention of LLINs.

MATERIALS AND METHODS

Study design, study areas and study period

Field- and laboratory-based cross-sectional descriptive study design was done. Wild blood fed main vector *An. dirus* and larvae were collected from Mudon Township, Mon State from June 2013 to December 2014. Bio-efficacy of repeated hand washed PermaNet 2.0, and PermaNet 3.0 were tested against *An. dirus* mosquito in laboratory of Medical Entomology Research Division, Department of Medical Research (DMR) during the study period.

Mosquitoes collection

Anopheles dirus mosquitoes from Mudon Township, Mon State were collected for 5 days by animal bait big net {(K-net=Kanda net) 330x330x220 cm made by Prof. Kanda, Japan}, indoor and outdoor human bait collection from 18:00 to 02:00 hours with WHO sucking tube by monthly field surveys. All collected mosquitoes were put in paper cups with glucose and moisture

was maintained by covering with water soaked damp towel. The research work was approved by the Ethical Review Committee of DMR.

Larval collection

Anopheles larvae were collected in and around 3 km away from study areas. Wells, water pools, water pockets, foot prints, creeks, water holding containers and paddy fields were examined and *Anopheles* larvae were collected. Collected larvae were put in labeled plastic bag individually given oxygen by oxygen pump and all plastic bags were carried to Medical Entomology Laboratory and all collected larvae were reared to till adult.

Species identification

Species identification of all collected *Anopheles* mosquitoes by different catching methods and adults emerged from larval survey were done according to different authors.^{12, 13}

Mosquitoes rearing

Blood fed all collected *An. dirus* from Kunkathaung Ward, Mudon Township, Mon State were oviposited and hatched larvae from eggs were reared continuously to get next generation of mosquitoes for bio-efficacy test with PermaNet 2.0 and 3.0.

Preparation of mosquito nets samples

LLINs description

PermaNet 2.0, a factory-treated mosquito net, made with netting material that has deltamethrin insecticide (55 mg/m²) incorporated within or bound around the fibres made of 100% polyester netting material and multiple wash-resistant resins. The net measuring 160x180x150cm comprised of 100 denier polyester yarn having 56 mesh/in² was a factory product manufactured by M/s Vestergaard Frandsen, Denmark.

PermaNet 3.0, combination net (Vestergaard Frandsen SA, Aarhus, Denmark) is a LLIN consisting of a top panel made of mono-filament polyethylene (100 denier) fabric

incorporating deltamethrin at 4 g/kg(121 mg/m²) and PBO at 25 g/kg(759 mg/m²), plus side panels made of multi-filament polyester (75 denier) fabric with a strengthened border coated with a wash-resistant formulation of deltamethrin at a target dose of 2.8 g/kg (85 mg/m²). The 75 denier sides of PermaNets 3.0 include a specially designed lower 70 cm border to enhance the lifetime of the net in the field. The net is made fabric material -WHOPES approved fabric 100% Polyester/polyethylene netting material and multiple wash-resistant and mainly improved for pyrethroid resistance *Anopheles* mosquitoes. The net measuring 190x180x150 cm, was a factory product.

Net samples

PermaNet 2.0 mosquito nets were provided from Vector Borne Diseases Control, Department of Health, Nay Pyi Taw and PermaNet 3.0 nets were provided from MAST Myanmar Technology Company Ltd., Yangon, Myanmar for bio-efficacy testing of each mosquito net.

Washing procedure

The bio-efficacy of each net (zero wash) was determined before washing by exposing 3-5 days old unfed females the susceptible *An. dirus* in WHO cone bioassays test.⁷ After testing of 0 wash each mosquito nets were washed by hand every 14th day up to 30 washed. Five grams of Fuji detergent cream were dissolved in 10 liters of tap water in 30 liters plastic bowl. Each net was soaked for 10 minutes. The soaked net was rubbed thoroughly between the palms for 15 minutes and rinsed in plain water 3 times to remove the detergent. After washing, the nets were shade-dried for 5-7 hours at 25-30°C depending on the month.

Mosquito susceptibility test

Before evaluation of bio-efficacy of nets, laboratory-reared 3-5 days old ten *An. dirus* each from Mudon colony was evaluated for insecticide susceptibility to different insecticides (DDT 4%, deltamethrin 0.05%, permethrin 0.75%, cyfluthrin 0.15% and PY

control papers) used for vector control to conform susceptible strain. Susceptibility test was performed using WHO impregnated papers and test kits.¹⁴

Three replicates were done for each test. According to WHO, 5-10 adult mosquitoes per tube or cone is enough for susceptibility and bio-efficacy tests.¹⁴ A total of 180 *An. dirus* mosquitoes {(10 mosquitoes x 6 (4 insecticide papers+1PY control+1OC control)=60 mosquitoes x 3 replicates=180} were used for testing against different insecticides impregnated papers. Mortality was confirmed after 24 hours of exposure time.

Bio-assay test for efficacy and wash resistance of washed nets

Three-minute cone bio-assay test was conducted after each wash of 0, 10, 20 and 30 washed PermaNet 2.0, and PermaNet 3.0 netting from the top roof and side of the nets were attached with WHO cones horizontally. Depending on availability of mosquitoes, 5 each 3-5 days old laboratory-reared unfed *An. dirus* from Mudon were introduced in cones by sucking tube and exposed for 3 minutes using WHO cone bioassay test method.⁷

This test consisted to expose female mosquitoes to each part of the nets. Two replicates were done to each category of the washed nets {(5 mosquitoes x 1 top roof) + (5 mosquitoes x 1 side)=10 x 5 nets (4 test nets+1 control net)=50 x 2 replicates =100 mosquitoes}). Therefore, 100 *An. dirus* mosquitoes were required every tenth washes of both LLINs and untreated control mosquito nets. The mosquitoes were then, removed from the cones and placed in paper cups with 10% glucose soaked cotton and moisture was maintained. Percentage of knockdown was measured after 60 minutes exposure and mortality was accessed 24 hours after exposure period.

Data analysis

Knockdown and mortality data were analyzed by Microsoft EXCEL software.

Data processing and analysis were conducted using appropriate statistical software such as EPIDATA and SPSS version 8 for entomological data.

RESULTS

A total of 7 *Anopheles* species comprising *An. maculatus*, *An. annularis*, *An. aconitus*, *An. minimus*, *An. vagus*, *An. philippinensis* and *An. dirus* were collected from Mudon Township during the study periods. *An. maculatus* was the predominant species accounting for 252 followed by *An. annularis* 126. The main vector *An. minimus* was observed 63 and *An. dirus* 184 during the study period. According to larva survey, *An. dirus* larvae were collected in domestic wells in high number and 60% of the wells were found positive for *An. dirus* larvae in rainy season although the larvae were found throughout the year in some vegetative wells. *An. annularis*, *An. minimus*, *An. vagus* and *An. maculatus* larvae were collected in water pools, sand pools, creeks and cow foot prints in and around the Conkathaung Village, Mudon Township. Sometimes, *An. maculatus* larvae were found together with the *An. dirus* larvae in wells.

Table 1. Susceptibility status of laboratory-reared *An. dirus* to different insecticides used in malaria control

WHO Insecticide impregnated paper	Exposure time (hr)	Knock-down(%)	24hr post-exposure mortality (%)
DDT4%	1	100	100
Deltamethrin 0.05%	1	100	100
Permethrin 0.75%	1	100	100
Cyfluthrin 0.15%	1	100	100
PY control	1	0	0
OC control	1	0	0

Table 1 shows susceptibility status of laboratory-reared *An. dirus* from Mudon train was tested by WHO test kit method with different WHO insecticides impregnated papers which are used in malaria control programmes. The results showed 100% knockdown and 100% mortality in all tested insecticide impregnated papers. In control

test, there was no knockdown, and mortality in any *An. dirus* mosquitoes within 24 hours. Laboratory-reared *An. dirus* were found highly susceptible to all tested insecticides.

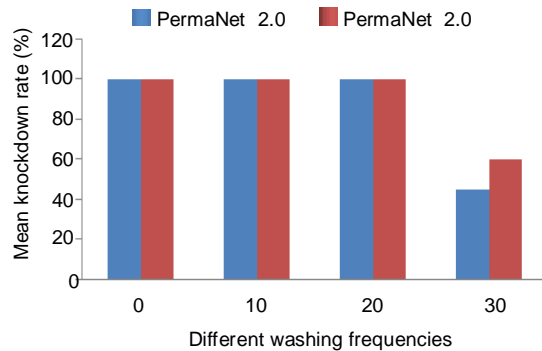


Fig. 1. Mean knockdown rate of susceptible *An. dirus* against before and after washing of PermaNet 2.0 and PermaNet 3.0 within 60 minutes in laboratory

Bio-efficacies of 20 washes PermaNet 3.0 was observed 100% knockdown and PermaNet 2.0 also induced 100% knockdown against laboratory-reared susceptible *An. dirus*. The efficacy significantly failed down to 60% knockdown for PermaNet 3.0 and 45% knockdown for PermaNet 2.0 after 30 washes (Fig. 1).

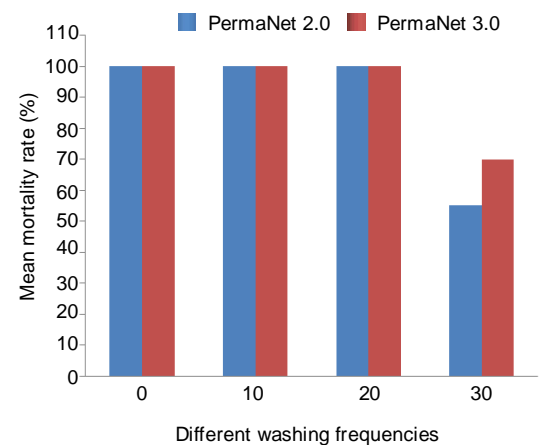


Fig. 2. Mean mortality rate of susceptible *An. dirus* against before and after washing of PermaNet 2.0 and PermaNet 3.0 within 24 hours in laboratory

Bio-efficacies of 20 washes PermaNet 2.0 and PermaNet 3.0 were found 100% mortality against laboratory-reared susceptible

An. dirus. The efficacy was significantly failed down to 70% mortality for Permanent 3.0 and 55% mortality for PermaNet 2.0 after 30 washes, respectively (Fig. 2).

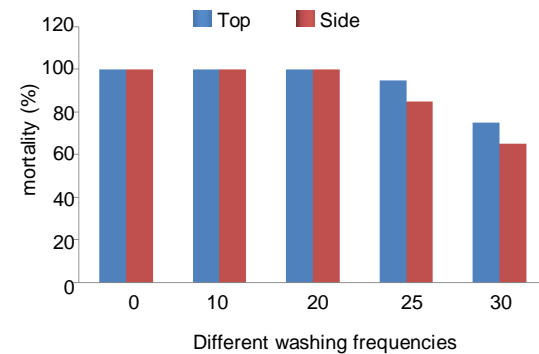


Fig. 3. Efficacy of PermaNet 3.0 top and side panels against susceptible *An. dirus* tested by WHO cone bioassay test method

The percentage mortality induced by PermaNet 3.0 against *An. dirus* differed between top roof and sides (Fig. 3). Efficacy of PermaNet 3.0 found 100% mortality after 20 washes. After 25 and 30 washes the bio-efficacy of top roof was found 95% and 75%, respectively. The side netting induced only 85% and 65% mortality after 25 and 30 washes, respectively. The top netting observed higher mortality rates than the sides: 100% at 0 wash, decreasing to 75% after 30 washes. The side bio-efficacy was mostly lost between 25 and 30 washes (Fig. 3).

DISCUSSION

A total of 7 *Anopheline* species were collected during the study period, *An. maculatus* was predominantly present in study area followed by *An. annularis*. The main vector *An. dirus* and *An. minimus* were observed 184 and 63 during the study period. *An. dirus* larvae were found a high number in domestic wells. Previously, a high number of *An. minimus* was found in foothill and forest fringe areas but now it is found in rice field in plain areas.¹⁵ *Anopheles dirus* species are also bred in deep forest areas but now their larvae were

found in men-made wells in Mon State and Thanintheryi Region,^{16, 17} because certain environmental changes like deforestation and vegetation clearance for crop plantations and also rapid growth of population and installation of new rural areas may lead to an increase abundance of mosquito larval habitats.¹⁸

Susceptibility status of laboratory-reared *An. dirus* from Mudon was tested by WHO cone bioassay test kit method with different WHO insecticides used for malaria control programmes. The result was found 100% knockdown and 100% mortality. Laboratory-reared *An. dirus* was found highly susceptible to all tested WHO insecticides impregnated papers as DDT 4%, deltamethrin 0.05%, permethrin 0.75%, cyfluthrin 0.15%. Susceptible main vectors are very useful for bio-efficacy testing of IRS, insecticide treated nets, and LLINs nets for determination of accuracy before and after washing of LLINs and ITNs nets. The high levels of insecticide resistance in *An. gambiae s.s* mosquitoes collected in West Africa area were confirmed by the low mortality rates in WHO susceptibility tests with DDT, deltamethrin and permethrin (2.9%, 10.6% and 43.9%) mortality, respectively.¹⁹ But in Myanmar, all collected main vectors *An. dirus* and *An. minimus* were 100% susceptible to DDT 4%, deltamethrin, permethrin and cyfluthrin.²⁰

Bio-efficacy of unwashed and after 20 washes PermaNet 2.0 and PermaNet 3.0 were found not significant difference between nets, 100% knockdown and 100% mortality against laboratory-reared susceptible *An. dirus* to PermaNet 2.0 and 100% knockdown and mortality to PermaNet 3.0. The insecticidal efficacies of both nets were very high in killing main vector *An. dirus* after 25 washed. Same result has been found in Zeneti village, Muheza District, NE Tanzania (5°13'S and 38° 39'E). Before washing of the PermaNet 2.0 and 3.0 nets, the percentage knockdown and mortality of *An. gambiae* Kisumu were recorded as 100% for each treatment

arm. After 20 washes, PermaNet 3.0 still scored 100% mortality whereas PermaNet 2.0 scored 96% mortality. At the end of the 36-day trial, mortality in PermaNet 3.0 and 2.0 arms stood at 96% or more and in the CTN washed to just before exhaustion mortality stood at 90%.²¹ A previous bio-efficacy study of LLIN and ITNs in Myanmar mentioned that PermaNet 2.0 is a very effective control tool for vector mosquitoes, with 100% knockdown and mortality against *An. dirus* and *An. minimus* before and after 20 washes. Same efficacy was found in K-O-Tab+binder treated net against *An. dirus* and *An. minimus* after 20 washes but *An. minimus* was found 100% mortality till 15 washes, and 86% mortality when tested against 20 washes net of K-O-Tab only treated net.²⁰ An intervention study in Thayetchaung Village, Bawbin Dam area found that conventionally deltamethrin-treated nets in regular use caused 100% mortality of malaria vector *An. minimus* and secondary vectors *An. vagus*, *An. maculatus*, *An. culicifacies* and *An. annularis* up to 6 months in 3 minutes bioassay.²²

A recent study carried out in Benin, West Africa, showed that although unwashed PermaNet® 3.0 was associated with a higher mortality than unwashed PermaNet 2.0 or unwashed Olyset, once washed, PermaNet 2.0 and PermaNet 3.0 performed similarly.²³ However, the present study found that both unwashed LLINs nets have similar bio-efficacy, 100% mortality till 20 washes on susceptible *An. dirus*. PermaNet 3.0 had higher mortality after 21 to 30 washes than PermaNet 2.0. In Vietnam where resistance did not seem to be affecting net efficacy, all PermaNet arms were performing slightly better than conventionally treated nets washed until just before exhaustion.²⁴ In Burkina Faso, in Vallee du Kou where the kdr mutation frequency was high (>80%), results showed a strong reduction of LLIN efficacy. In this area, a significantly higher mortality and blood feeding inhibition was associated with unwashed and

washed PermaNet® 3.0 compared to unwashed and washed PermaNet® 2.0.⁴ Elevated P450 activity in a strain of *An. gambiae* from Cameroon has been found to reduce the efficacy of permethrin-treated netting in laboratory tests.²⁵

In the present study, the efficacy was found significantly failed down to 60% knockdown and 70% mortality for PermaNet 3.0 and 45% knockdown and 55% mortality for PermaNet 2.0 after 30 washes. The mortality rate against *An. gambiae* in Kisumu was greater with PBO-deltamethrin netting than with deltamethrin netting ($p=0.04$). Washing the two types of netting up to 20 times do not have a significant effect on mortality of the highly susceptible *An. gambiae* Kisumu.²³

The percentage mortality induced by PermaNet 3.0 against the *An. dirus* differed between top roof and sides (Fig. 3). Efficacy of PermaNet 3.0 found 100% mortality after 20 washes. Regarding the panels, the bioefficacy of top panel was found 95% and 75% after 25 and 30 washes, respectively. The side netting affected only 85% and 65% mortality after 25 and 30 washes. The top netting induced higher rates of mortality than the sides: 100% at 0 wash, decreasing to 75% after 30 washes ($p=0.01$).

The side mostly lost its efficacy between 25 and 30 washes. A study in Southern Berlin revealed that the mean loading dosage of deltamethrin on the roof (PermaNet 3.0) showed only a slight decrease after 20 washes from 119 mg/m to 101 mg/m² but that of the deltamethrin on the sides 94 mg/m² had fallen to 17 mg/m² after 20 washes. This is due to the deltamethrin on the roof being incorporated into the polyethylene and thus less tractable to washing. It was recorded that PBO on the roof of the net was 731 mg/m² and 856 mg/m² before washing (two nets tested), at 435 mg/m² after washing and at 258 mg/m² by the end of the trial. Deltamethrin retention after 20 washes

was 18% on the sides and 84% on the roof.²³

The unwashed PermaNet 3.0 showed no difference to unwashed PermaNet 2.0 against pyrethroid-susceptible *An. dirus*, in terms of mortality (100% killed). After 20 washes the two products showed no difference in knockdown and mortality (100% with PermaNet 2.0 and 100% with PermaNet 3.0) but showed small differences in knockdown and mortality after 30 washes (45%, 60% and 55%, 70% with both PermaNet 2.0 & 3.0, respectively).

A study in Assam India mentioned that PermaNet 2.0 nets had reasonably good wash resistance for up to 15 washes against *An. minimus* s.l, and reduced the biting densities of culicine in houses and inhibited blood feeding by mosquitoes.⁵ But, for protecting against *An. dirus*, PermaNet 3.0 showed no difference to PermaNet 2.0 when either unwashed or after 20 washes, according to the WHO cut-off point of 95% knockdown and 80% mortality.⁷ Both products were highly protective against biting. Laboratory tunnel bioassays confirmed the loss of biological activity of the PBO/deltamethrin-treated panel after washing.²¹

Conclusion

Both PermaNet products are highly effective against susceptible *Anopheles dirus*. Both PermaNet 2.0 and PermaNet 3.0 have good wash resistance for up to 20 washed 100% mortality against *An. dirus* which is the major vector of malaria in Myanmar. PermaNet 3.0 is improved as a long lasting insecticidal net (LLIN) to control or protect against pyrethroid-resistant mosquitoes. Large-scale studies are needed to confirm its insecticidal efficacy and durability against different malaria vector species in field areas where main vector and malaria incidence are endemic.

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