BIOEFFICACY OF A LONG-LASTING INSECTICIDE IMPREGNATED NET ON BLOOD FEEDING INHIBITION OF *ANOPHELES MACULATUS* THEOBALD AND *CULEX QUINQUEFASCIATUS* SAY

Tan Swee Beng, Indra Vythilingam and Lee Han Lim

Medical Entomology Unit, WHO Collaborating Centre for Vectors, Institute for Medical Research, Kuala Lumpur, Malaysia

Abstract. This study aimed to evaluate the bioefficacy and blood feeding inhibition of mosquitoes under laboratory conditions using the WHO tunnel test method on unwashed and washed long-lasting insecticide impregnated net with extrinsic heat treatment of 30ºC followed by 80ºC on the same net during washing. PermaNet® exhibited fairy high durability to washing (5 washes) and had fairy long-lasting bioefficacy against *Anopheles maculatus* for blood feeding inhibition on both unwashed (39 months) and washed (26 months) nets. However, PermaNet exhibited lower bioefficacy against *Culex quinquefasciatus.* This study also suggested that the application of extrinsic heat treatment of 30ºC followed by an increased heat at 80ºC on the same net exerted significant differences ($p<0.05$) in mortality of both *An. maculatus* and *Cx. quinquefasciatus.* However, extrinsic heat treatment did not enhance any significant increase in blood feeding inhibition of both *An. maculatus* and *Cx. quinquefasciatus* ($p>0.05$). *An. maculatus* exhibited significant differences in resting preference after a successful blood meal, as more blood-fed and live females preferred to rest and stay near the bait in the mouse cage, and more dead and unfed females were found in the outer cage of both the unwashed and washed nets ($p<0.05$). Conversely, fully blood-fed and live *Cx. quinquefasciatus* females did not show any resting preference between the mouse cage and outer cage, but there were more dead and unfed females in the mouse cage of both the unwashed and washed nets ($p<0.05$).

Keywords: *Anopheles maculatus* Theobald, *Culex quinquefasciatus* Say, blood-feeding inhibition, extrinsic-heat treatment, PermaNet®, tunnel mortality

INTRODUCTION

The concept of impregnation of bednets with an insecticide is based on the fact that mosquitoes alighting on the net will be killed on contact thereby reducing man/vector contact and overall vector density (Vythilingam *et al,* 1996; Curtis *et al*, 2003). Insecticide-treated bed nets (ITNs) are now a major intervention for malaria control (CDC, 2012). It is reported that treated nets continue to have a powerful impact on vector populations (Curtis *et al*, 2003). PermaNet®, a long-lasting insecticide-treated net (LLIN), is pretreated in a factory with deltamethrin, a pyrethroid,

Correspondence: Dr Lee Han Lim, Medical Entomology Unit, WHO Collaborating Centre for Vectors, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia.
Tel/Fax: +60 3 2616 2688
E-mail: leehl@imr.gov.my

which is a neurological insecticide that is rapidly absorbed by the legs of the mosquito while in contact with the insecticide treated nets. Deltamethrin is also an excito-repellent insecticide which affects insect behavior including feeding inhibition as mosquitoes that stay in the room to rest are stimulated to leave (WHO, 1990; 2012). The presence of pyrethroid on a net greatly reduces susceptible mosquito's ability to feed through the fabric or penetrate small gaps in it (Curtis *et al*, 1991; Lengeler *et al*, 1996; Heierli and Lengeler, 2008). Studies have shown that an insecticide-treated net with large holes protected and reduced biting by up to 95% (Lines *et al,* 1987; Lindsay *et al,* 1991; Miller *et al*, 1991; Curtis *et al*, 1992; Pleass *et al*, 1993; Lengeler *et al* 1996; Heierli and Lengeler, 2008; Irish *et al,* 2008). Recent studies done by Gnanguenon *et al* (2013) showed that at a proportionate hole index (pHI) of 276, man-vector contact was observed in torn LLINs indicating that the insecticide at the surface of LLINs could only reduce the number of vectors. Ochomo *et al* (2013) and Gnanguenon *et al* (2013) showed that in areas with pyrethroid resistant vectors, LLINs with modest holes permit mosquito entry and feeding, and so providing little protection against these vectors. Deltamethrin is more toxic to mammals than permethrin, but its toxicity to insects, even in low concentrations compensates for this (CDC, 2012). Therefore, as little as 10-25mg/m^2 is needed for the impregnation of bednets (WHO, 1990, 2012). Deltamethrin is insoluble in water, has little tendency for bioaccumulation in organisms, and is rapidly broken down in both soil and sunlight (WHO, 1990, 2012). Owing to its small quantity and good adhesion abilities to substrate, the residual activity of nets treated with deltamethrin can persist for 3-5 years, even with 20 washings (WHO, 2013).

Because the cone bioassay test does not provide the overall insecticide bioefficacy under field conditions due to the forced tarsal contact that does not allow natural avoidance behavior of the adult mosquitoes, therefore, the tunnel test was developed to better stimulate field conditions (WHO, 2005).

The objective of this study was to evaluate the bioefficacy and blood feeding inhibition of mosquitoes under laboratory conditions on unwashed and washed nets with extrinsic heat at 30ºC followed by 80ºC on the same nets during washing.

MATERIALS AND METHODS

Study site

The study was conducted from 23 July 2007 until 28 March 2010 in the main laboratory of the Medical Entomology Unit/WHO Collaborating Centre for Vectors, Infectious Disease Research Centre, Institute for Medical Research, Kuala Lumpur. The temperature and relative humidity were maintained at 27±2ºC and 80±2%, respectively with a photoperiod of 12:12 hours (L:D).

Mosquito nets used in the study

PermaNet (Vestergaard Frandsen; Lausanne, Switzerland), a second-generation product, was made of 75 denier polyester with a mesh size of 25 holes/cm^2 and treated in the factory with 55mg/m^2 deltamethrin. The size of the net was 160 cm x 150 cm x 180 cm (width x height x length). A similar but untreated net was also tested as control net. Four samples of the net (25 x 25 cm) were cut from each sides of the net and used in the testing.

Test mosquitoes

The mosquitoes used in the study were non-blood-fed 5-8 days old labora-

tory-bred *Anopheles maculatus* Theobald and *Culex quinquefasciatus* Say that were susceptible to insecticides including pyrethroids. The mosquitoes were fed with 10% sucrose solution fortified with 1% vitamin B complex and maintained in the insectarium at 27±2ºC and 80±2% RH with 12:12 hours (L:D) photoperiod regime.

Laboratory washing procedure

The net samples (25 cm x 25 cm) were individually introduced into 1 liter beakers containing 0.5 liter distilled water, with 2 g/l soap (pH 10-11) fully dissolved. The beakers were then placed into a shaking water bath at 30ºC and 80ºC, respectively and shaken for 10 minuted at 155 rotations per minute. The net samples were then removed and rinsed twice for 10 minutes in clean distilled water in the same shaking condition as stated above. After washing and drying at room temperature, the net samples were tested to determine effectiveness soon after washing. In this trial, the same nets were washed three times at 30ºC followed by two final washes at 80ºC and shaken for 10 minutes at 155 rotations per minute.

Tunnel test

The basic equipment (Fig 1) consisted of a square section netting tunnel of 25 cm x 25 cm, 75 cm long derived from Elissa and Curtis (1995) as described in Chandre *et al* (2000). At one-third of the length, a disposable cardboard frame was placed with the treated sample. The surface of the net in contact with the mosquitoes was 20 cm x 20 cm with nine exit holes (1 cm diameter) precisely positioned. Inside the small part of the tunnel (the mouse cage), bait (a white mouse) was placed, restrained but available for biting. At the end of each side of the tunnel, a 30 cm square cage was fitted and covered with polyester netting. In the cage placed at the end of the large part of the tunnel (the outer cage), 100 starved, 5-8 days old *An. maculatus* female adults were introduced at 4:30 PM. Females were free to fly in the cage, but they had to be in contact with treated netting and locate the holes before reaching the bait.

The following morning at 7:00 AM, the females were removed and counted separately from each side of the cage. Immediate mortality for females that were dead unfed, dead blood-fed, live unfed, and live blood-fed were recorded. Live females were placed in paper cups with 10% sugar solution fortified with 1% vitamin B complex and delayed mortality after 24 hours was recorded.

Three tunnels were used simultaneously, of which one served as a control. The same testing procedures were applied to *Cx. quinquefasciatus.* Blood-feeding inhibition was analyzed by comparing the proportion of blood-fed females in treated and control tunnels. Overall mortality was measured by pooling the immediate and delayed mortalities of mosquitoes from the two sections of the tunnels (mouse cage and outer cage). The total numbers of mice used for testing *An. maculatus* and *Cx. quinquefasciatus* were 43 and 29, respectively.

Ethical considerations

Approval to use mice for entomological research was obtained from the Ministry of Health [Ref Nº KKM.KPK.5305.20/11 Jld 13(37), 2006 Nov 24], in accordance with the Destruction of Disease Bearing Insects Act, 1975.

Data analysis

The numbers of dead mosquitoes were recorded. The mortalities were corrected using Abbott's formula (Abbott, 1925) if the control mortality was <10% (WHO, 2013):

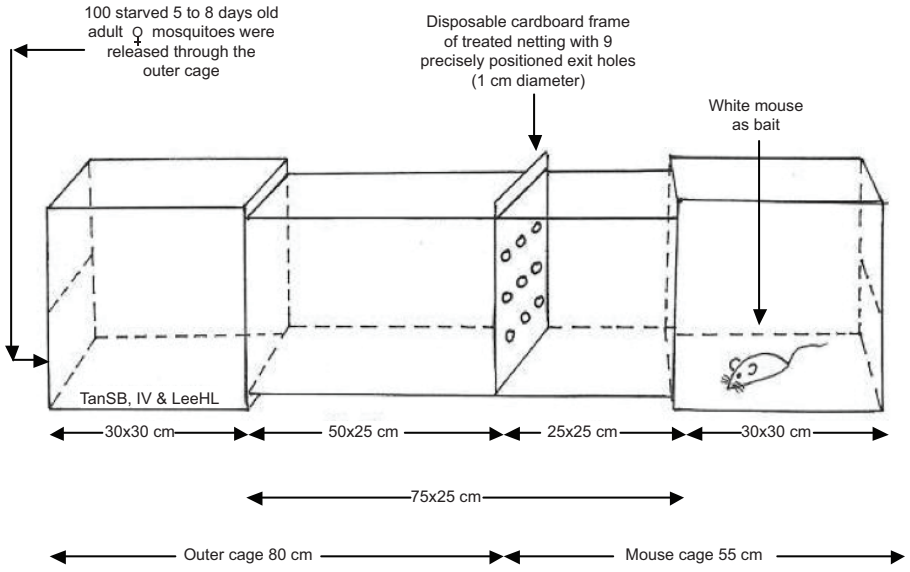

Fig 1–Polyester netting tunnel.

$$\frac{\%\ \text{treated mortality} - \%\ \text{control mortality}}{100\% - \%\ \text{control mortality}} \times 100\%$$

When results obtained in test where control mortalities exceeded 10%, the test was discarded and the experiment repeated (WHO, 2013). All data were expressed as the mean ±SD. A value of $p<0.05$ was considered significant. Data obtained from the test were subjected to statistical analysis using a statistical software program (SPSS® version 16.0; IBM, Armonk, NY).

RESULTS

In the unwashed nets tested against *An. maculatus*, only 3.60±2.79% (Table 1; Fig 2) females were found to be blood-fed and alive in the mouse cage with a mean mortality of 74.36±25.77% (Table 3; Fig 3) over a 39-month test period. Compared to control net, there were 17.00±7.81% (Table 1; Fig 2) blood-fed and live females together with a total of 84.25±18.84 unfed and live females where 15.33±14.74% of them were found in the mouse cage and 72.75±8.38% found in the outer cage (Table 2).

In the washed nets, only a total of 4.00±2.83% females were blood-fed and alive, whereas 2.50±0.71% blood-fed and live females were found in the mouse cage, and 3% blood-fed and live females were found in the outer cage (Table 1; Fig 2) with a mean mortality of 75.00±18.46% over a 26-month test period (Table 3; Fig 3). As compared to the control net, 26.50±36.06% (Table 1; Fig 2) blood-fed and live females together with 74.50±21.33% (Table 2) unfed and live females where 20.00±26.87% (Table 1; Fig 2) of the blood-fed and live females together with 24.00±12.08% (Table 2) unfed and live females were found in the mouse cage, and 13.00% (Table 1; Fig 2) blood-fed and

Table 1
Blood feeding inhibition of *An. maculatus* on unwashed and washed PermaNet.

Treated net	Months	*n*	Blood-fed (%)	*p*-value
Overall unwashed	39	5	3.60±2.79	0.045
Overall unwashed (control)	39	3	17.00±7.81	0.064
Overall washed 30ºC and 80ºC	26	2	4.00±2.83	0.295
Overall Washed 30ºC and 80ºC (control)	26	2	26.50±36.06	0.488
Overall unwashed mouse cage	39	5	3.60±2.79	0.045
Overall unwashed mouse cage (control)	39	3	17.00±7.81	0.064
Overall unwashed outer cage	39	5	0	Nil
Overall unwashed outer cage (control)	39	3	0	Nil
Overall washed mouse cage	26	2	2.50±0.71	0.126
Overall washed mouse cage (control)	26	2	20.00±26.87	0.484
Overall washed outer cage	26	1	3	[a]
Overall washed outer cage (control)	26	1	13	[a]
3 washes at 30ºC	at 18th-21st	3	2.67±0.58	0.015
3 washes at 30ºC (control)	at 18th-21st	3	17.67±19.43	0.256
2 washes at 80ºC	at 22nd-26th	3	0	Nil
2 washes at 80ºC (control)	at 22nd-26th	3	0	Nil
Unwashed/washed		20		<0.050
Mouse cage/outer cage (Resting preference after a blood meal)		7		<0.050

[a]Cannot be computed because the sum of case weights is ≤1.

Table 2
Unfed and live *An. maculatus* on control nets of unwashed and washed PermaNet.

Control net	Months	*n*	Unfed (%)	*p*-value
Overall unwashed	39	4	84.25±18.84	0.003
Overall unwashed mouse cage	39	3	15.33±14.74	0.213
Overall unwashed outer cage	39	4	72.75±8.38	0.000
Overall washed	26	6	74.50±21.33	0.011
Overall washed mouse cage	26	5	24.00±12.08	0.001
Overall washed outer cage	26	6	54.50±18.31	0.000

live females and 54.50±18.31% (Table 2) unfed and live females were found in the outer cage.

In the unwashed nets tested against *Cx. quinquefasciatus*, there were 34.64 ±21.15% blood-fed and live females, whereas 32.83±5.85% blood-fed and live females were found in the mouse cage, and 8.83±7.39% blood-fed and live females were found in the outer cage (Table 4; Fig 4) with a low tunnel mortality of 44.90±11.98% over the test period of 13 months (Table 6; Fig 5). Compared to the control net, there were 79.67±13.81% blood-fed and live females, whereas 64.00±11.36% (Table 4; Fig 4) blood-fed and live females together with 1.50±0.71% (Table 5) unfed and live

Table 3
Mortality of *An. maculatus* on unwashed and washed PermaNet.

Treated net	Months	*n*	Mortality (%)	*p*-value
Overall unwashed	39	14	74.36±25.77	0.000
Overall unwashed mouse cage	39	8	23.50±17.85	0.007
Overall unwashed outer cage	39	8	43.50±23.22	0.007
Overall washed 30ºC and 80ºC	26	10	75.00±18.46	0.000
Overall washed 30ºC and 80ºC mouse cage	26	10	23.60±14.49	0.007
Overall washed 30ºC and 80ºC outer cage	26	10	50.80±15.21	0.000
3 washes 30ºC	at 18^{th}-21^{st}	6	74.67±24.66	0.001
2 washes 80ºC	at 22^{nd}-26^{th}	4	75.50±3.00	0.000
Unwashed/washed mortality	39/26	14/10		<0.050
Mouse cage/outer cage mortality	39	8/10		>0.050

Table 4
Blood feeding inhibition of *Cx. quinquefasciatus* on unwashed and washed PermaNet.

Treated net	Months	*n*	Blood-fed (%)	*p*-value
Overall unwashed	13	14	34.64±21.15	0.000
Overall unwashed (control)	13	6	79.67±13.81	0.000
Overall unwashed mouse cage	13	6	32.83±5.85	0.000
Overall unwashed mouse cage (control)	13	3	64.00±11.36	0.010
Overall unwashed outer cage	13	6	8.83±7.39	0.033
Overall unwashed outer cage (control)	13	3	20.67±11.15	0.085
1 washed 30ºC	1	4	47.25±10.75	0.003
1 washed 30ºC (control)	1	4	47.25±10.75	0.000
1 washed 30ºC mouse cage	1	4	46.75±5.25	0.000
1 washed 30ºC mouse cage (control)	1	2	6.50±2.12	0.017
1 washed 30ºC outer cage	1	4	19.00±8.87	0.023
1 washed 30ºC outer cage (control)	1	2	38.00±0.00	0.000
Unwashed/washed		16		>0.050
Mouse cage/outer cage (Resting preference after a blood meal)		8		>0.050

females were found in the mouse cage, and 20.67±11.15% (Table 4; Fig 4) blood-fed and live females and 5.33±4.04% (Table 5) unfed and live females were found in the outer cage.

In the washed nets (two washes at 30ºC), there were 65.75±11.5% blood-fed and live females, whereas 46.75±5.25% blood-fed and live females were found in the mouse cage, and 19.00±8.87% blood-fed and live females found in the outer cage (Table 4; Fig 4), with a low tunnel mortality of 35.25 ±12.20% over the test period of 4 months (Table 6; Fig 5). Compared to the control net, there were 47.25±10.75% blood-fed and live females, whereas 6.50±2.12% (Table 4; Fig 4) blood-fed and live females together with

Table 5
Unfed and live *Cx. quinquefasciatus* on unwashed and washed PermaNet.

Treated net	Months	*n*	Unfed (%)	*p*-value
Overall unwashed	13	3	6.33±3.79	0.101
Overall unwashed mouse cage	13	2	1.50±0.71	0.210
Overall unwashed outer cage	13	3	5.33±4.04	0.150
Overall washed	1	1	3.00	[a]
Overall washed mouse cage	1	1	3.00	[a]
Overall washed outer cage	1	1	6.00	[a]

[a]Cannot be computed because the sum of case weights is ≤1.

Table 6
Mortality of *Cx. quinquefasciatus* on unwashed and washed PermaNet.

Treated net	Months	*n*	Unfed (%)	*p*-value
Unwashed	13	10	44.90±11.98	0
Overall unwashed mouse cage	13	6	34.83±9.85	0
Overall unwashed outer cage	13	6	14.67±9.35	0.012
Overall unwashed outer cage	13	6	14.67±9.35	0.012
1 wash 30ºC	1	4	35.25±12.20	0.010
1 washed 30ºC mouse cage	1	4	16.50±9.85	0.044
1 washed 30ºC outer cage	1	4	13.75±3.20	0.003
Unwashed/washed mortality	13	10		<0.050
Unwashed/washed mortality	13	10		<0.050
Mouse cage/outer cage mortality	13	10		<0.050

3% (Table 5) unfed and live females were found in the mouse cage and 38.00±0.00% (Table 4; Fig 4) blood-fed and live females and 3% (Table 5) unfed and live females were found in the outer cage. Because mortality was so low, no further washing at 80ºC on the same net was carried out.

DISCUSSION

There were significant differences in mortality between unwashed and washed PermaNet for both *An. maculatus* and *Cx. quinquefasciatus* ($p<0.05$). PermaNet exhibited fairy high durability to washing (five washes) and had fairly long lasting bioefficacy against *An. maculatus*. These results concurred with the findings of Kilian *et al* (2008) where mortality and knockdown of susceptible *An. gambiae* s.s. remained high (88.5% and 97.8%, respectively) after 36 months of regular household use in rural Uganda.

A study performed against *An. stephensi* showed 93.8% and 50% mortality, respectively, and against unwashed and 6-times washed deltamethrin-treated nets (ITN) (Rafinejad *et al*, 2008). This study also showed that the mortality rate of *An. stephensi* exposed to PermaNet (LLIN) decreased from 100% on unwashed net to 92% after 20 washes. A study on an experimental hut done by Tungu *et al* (2010) indicated that both PermaNet 2.0 and 3.0 exhibited 95% mortality (unwashed), and

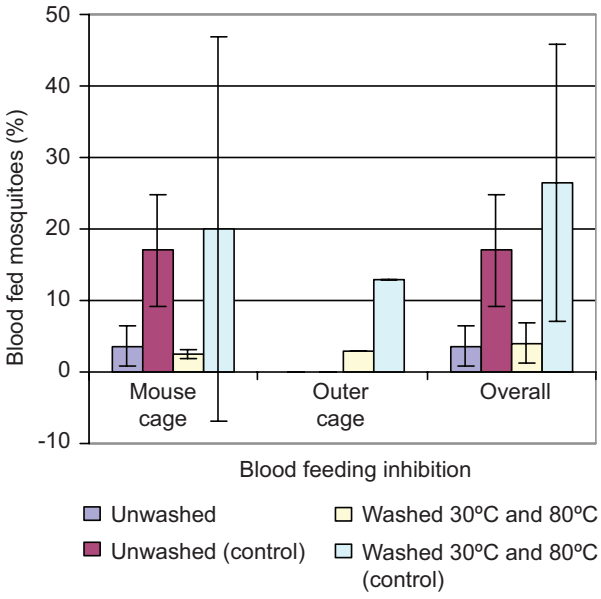

Fig 2–Blood feeding inhibition of *An. maculatus* on unwashed and washed PermaNet.

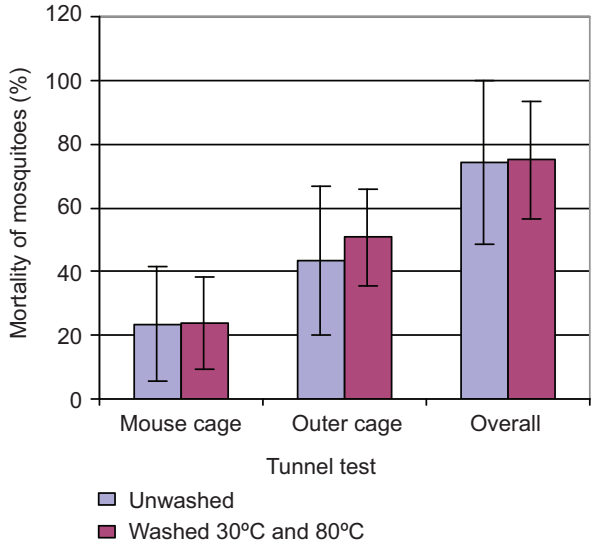

Fig 3–Mortality of *An. maculatus* on unwashed and washed PermaNet.

87% (20 washes PermaNet 2.0), and 95% mortality (20 washes PermaNet 3.0). A study done by Atieli *et al* (2010) on PermaNet 2.0 against *An. gambiae* s.l. reared from wild strains showed that after 15 washes, 50% of the fed died after 24 hours.

The present study also suggested that application of extrinsic heat treatment of 30ºC followed by an increased heat treatment of 80ºC on the same nets significantly increased tunnel mortality of *An. maculatus* ($p<0.05$). At 30ºC mortality was 74.67±24.66% and at 80ºC mortality increased to 75.50±3.00%.

PermaNet exhibited significant differences ($p<0.05$) on blood feeding inhibition against *An. maculatus* on both unwashed and washed nets. Our findings concurred with that of an experimental hut study conducted on PermaNet 2.0 and 3.0 where 10% and 3% (unwashed), and 9% and 10% (20 washes) of pyrethroid susceptible *An. gambiae* were blood-fed, respectively (Tungu *et al*, 2010).

Low blood feeding activity in the treated nets (3.60±2.79%) was observed in *An. maculatus*. This may be due to the natural avoidance behavior of the adult mosquitoes, because a tunnel test provides more space for flying and resting, and only a limited area of the treated net is placed at one side of the tunnel. This gives preference for resting due to the high irritant effect, which can considerably reduce the tarsal contact on the treated netting materials (Vythilingam *et al*, 1996; Hougard *et al*, 2003).

Our results indicated that the blood-fed and live females of *An. maculatus* preferred to rest and stay in the mouse cage of unwashed and washed nets after a successful blood meal ($p<0.05$). In 2008, Rafinejad *et al* mentioned that the test situation of tunnel test is close to the natural circumstances, but

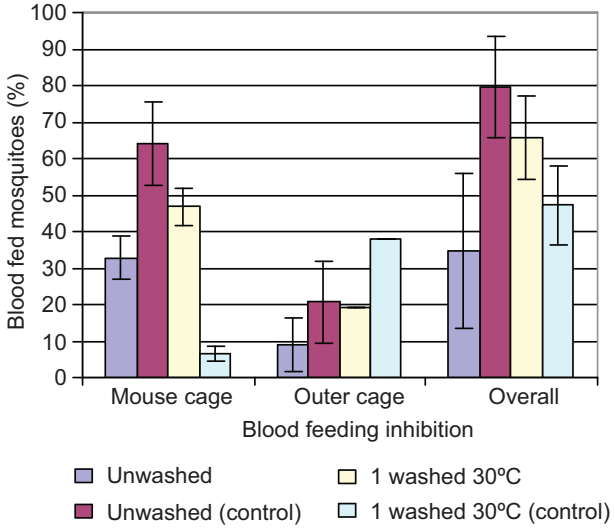

Fig 4–Blood feeding inhibition of *Cx. quinquefasciatus* on unwashed and washed PermaNet.

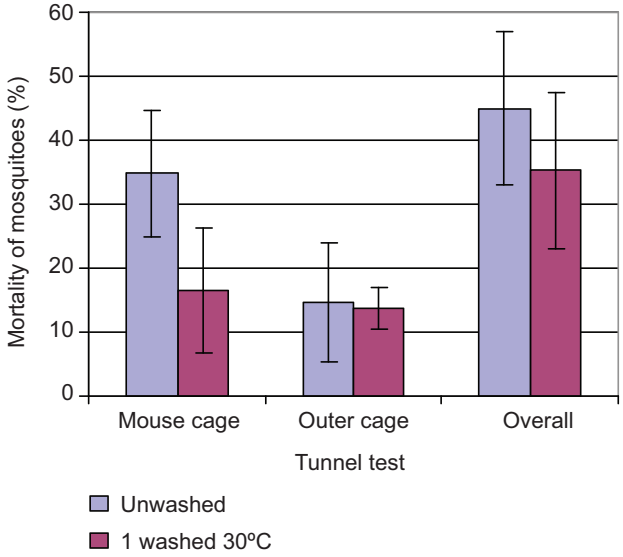

Fig 5–Mortality of *Cx. quinquefasciatus* on unwashed and washed PermaNet.

the mosquitoes may rest anywhere in the compartments as well as the sample nets. Our findings suggested that this may be true for *Cx. quinquefasciatus* but not for *An. maculatus*, probably due to different behaviors of the mosquitoes.

Blood feeding inhibition shown in the tunnel test indicated that female *An. maculatus* gained entry into the mouse cage and were killed without having a blood meal. This phenomenon suggests that deltamethrin impregnated nets have an inhibitory property on blood-feeding behavior of the females for eggs development, and that the excito-repellent and irritant effects of the insecticide caused the females to stay away from the treated nets (WHO, 2005).

The presence of a pyrethroid on a net greatly reduces a mosquito's ability to feed through the fabric or penetrate small gaps in it (Curtis *et al*, 1991; Lengeler *et al*, 1996; Heierli and Lengeler, 2008). Our study also suggested that insecticide treated nets are good even if torn because mosquitoes enter to feed but are unable to find their way out and thus are finally killed.

PermaNet exhibited lower bioefficacy on unwashed nets and did not inhibit blood feeding on unwashed nets over the test period of 13 months against *Cx. quinquefasciatus*. The low mortality of 35%-45% and a high blood-feeding rate of about 47%-66% indicated that the PermaNet was not effective against these mosquitoes. Most females were being attracted for a blood meal by the bait in the mouse cage and were fully blood-fed and alive. This study also indicated that there were no significant differences on blood-fed and live *Cx. quinquefasciatus* found between unwashed and washed nets. There was no significant resting preference of the females after a blood meal between mouse cage and outer cage of both the unwashed and washed nets. This

may be because the treated net was less lethal to *Cx. quinquefasciatus* than *Anopheles* species, although they were effective at preventing *Cx. quinquefasciatus* from biting (Curtis *et al*, 1991, 1994; Magesa *et al*, 1991; Kulkarni *et al*, 2007). Another reason may be due to the high irritant effect of deltamethrin (Potikasikorn *et al,* 2005; Muenworn *et al,* 2006; Sathantriphop *et al*, 2006) and the natural avoidance/escape behavior of these adult mosquitoes (Vythilingam *et al*, 1996; Chareonviriyaphap *et al,* 2004; Muenworn *et al,* 2006).

Long-lasting insecticidal net has a major role in the control of malaria. In 2014, about 200 million LLINs have been funded for delivery to endemic countries compared to 136 million in 2013 and 70 million in 2012 (WHO, 2014). When insecticide impregnated bednets are used over a wide area against vector of malaria, susceptible female mosquitoes risk death whenever they try to feed, and as such, the density of the local mosquito population including the parous rate and the sporozoite rate may drop (Curtis *et al*, 2003). Such effects were seen in village-scale trials in Assam and Tanzania (Curtis *et al*, 2003), Uganda (Kilian *et al*, 2008), Bungoma (Ochomo *et al,* 2013) and Ethiopia (Anshebo *et al,* 2014). Studies showed that after bednets were impregnated with deltamethrin, the mosquitoes resting on the surface of the bednets decreased significantly, although there was less effect on the total vector population (Atieli *et al*, 2010; Gnanguenon *et al*, 2013). Nets with strong excito-repellant properties may provide good protection from malaria, even if they do not effectively kill mosquitoes (WHO, 2005; Atieli *et al*, 2010).

In conclusion, tunnel testing of treated net is a very useful evaluation tool because it simulates the real life situation compared to bioassays.

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