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Laboratory Efficacy Tests of Pyrethroid-Treated Bed Nets on the Malaria Vector Mosquito, *Anopheles stephensi*, in a Baited Excito-Repellency Chamber

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Abstract: Insecticide-treated net is currently the best available method to control malaria. The extensive use of pyrethroid insecticides and the challenges of mosquito resistance to these chemical compounds are the main reasons for undertaking this study. The excito-repellency impacts of three different concentrations of three synthetic pyrethroid insecticide (lambdacyhalothrin, deltamethrin and cyfluthrin) impregnated bednets were evaluated against the susceptible and endophilic primary malaria vector, *Anopheles stephensi* Liston (Diptera: Culicidae) India susceptible strain under laboratory conditions. Young unfed female adult mosquitoes were exposed to animal bait covered with net in a dark exposure chamber. For each test, the results of mosquitoes' behavior were recorded after half an hour as dead, survived, blood-fed, recovered and retrieved in the exit trap. These studies clearly showed that populations of malaria vectors can be effectively controlled by the use of pyrethroid-treated bednets. The results inferred that deltamethrin was partially superior to other insecticides in terms of toxicity and revealed that cyfluthrin was clearly least effective and deltamethrin was most effective. The latter was 1.6 and 2.0 times more effective than lambdacyhalothrin and cyfluthrin, respectively, in killing *An. stephensi* mosquitoes. In addition, the mean recovery rate due to deltamethrin was 3.8 and 2.4 times less effective than cyfluthrin and lambdacyhalothrin, respectively. In conclusion, these data ranked the relative potency of the three pyrethroids in the order deltamethrin > lambdacyhalothrin > cyfluthrin.

Key words: *Anopheles stephensi*, excito-repellency, baited ITN chamber, deltamethrin, cyfluthrin, lambdacyhalothrin

INTRODUCTION

Malaria is a paradigm of an emerging malady. This is the most important vector-borne parasitic disease of man in most countries of the tropical world (WHO, 1995). Between 0.7-2.7 million people, mostly young children, die annually from malaria worldwide (Breman, 2001). Malaria is unstable (stability index <0.5) in Iran and about 14000 cases were reported in the year 2004. Vector control is a crucial component of malaria control programs. In fact, three of the four basic technical elements outlined in the Global Malaria Control Strategy by WHO (1995) involve vector control.

In many areas of the Eastern Mediterranean region and the Indian subcontinent, *Anopheles stephensi* Liston, 1901 is a primary vector of malaria (Glick, 1992). This is a peridomestic and largely endophilic mosquito species that is responsible for the transmission of malaria over a wide geographical range of Iran (Zaim and Cranston, 1986). It mates in a confined space and can thus be reared in the laboratory easily (Service, 1993). It is mainly recognized among others with three dark spots on its anal wing vein (Glick, 1992).

Insecticide-treated net (ITN) is currently the best available method to combat malaria. It is cheap, requires relatively low treatment level and uses low toxicity insecticides. Moreover, the efficacy of treated nets could be more than twice that obtained by untreated nets (Guyatt and Snow, 2002). More than a hundred trials and descriptive studies performed in all type of malaria settings globally have asserted the positive impact of ITNs on child and adult morbidity and mortality (Lengeler, 2004).

The expression of resistance to insecticides in anopheline mosquitoes is threatening to affect gravely the reemergence of vector-borne diseases like malaria

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(Krogstad, 1996) as well as disease control activities (Brogdon and McAllister, 1998). Indeed, the higher levels of resistance are reported from regions of ongoing control activities (Curtis, *et al*., 1998). There have been setbacks to malaria control efforts in Iran where anopheline resistance to insecticides has occurred (Gilles, 1993). The recent evolution and spread of mosquitoes resistant to the pyrethroid insecticides is threatening to reduce the potency of nets and to undermine present gains. As net usage increases, further selection of pyrethroid resistance seems inevitable. A knock-down pyrethroid resistance (kdr) mechanism involving a single A-T base change encoding a leucine to phenylalanine amino acid substitution is present in the pyrethroid-resistant *An. stephensi* strain (Enayati *et al*., 2003). Furthermore, no new classes of insecticides have been registered for vector control in over two decades (Zaim and Guillet, 2002).

Excito-repellency is a combination of contact irritability and non-contact repellency behavioural responses. The extensive use of pyrethroid insecticides and the challenges of mosquito resistance to these chemical compounds are considered the *a priori* reasons for undertaking these tests. The principal objective of this investigation was to deplore for the first time under laboratory conditions the efficacy of three pyrethroid insecticide-impregnated bednets against the primary malaria vector, *An. stephensi*, in a baited excito-repellency test chamber.

MATERIALS AND METHODS

Test population: Experiments were carried out on *Anopheles stephensi* India (BEECH) strain. This colony has been maintained in laboratory stocks at TUMS since 1996. This strain, originally collected in India in 1946, is used as a susceptible strain in most insectaries of Iran. Adult, unfed, 5-7 day old female *An. stephensi* mosquitoes were routinely employed.

Cage design: Excito-repellency test cage of Evans (1993) and Das (1997) were slightly modified for insecticide sensitivity studies. The improved version of the excito-repellency (E-R) test system is shown in Fig. 1. The exposure chamber is constructed with six aluminum sides, each side wall measuring 30×30 cm^2, forming a cube. In order to allow sample mosquitoes delivery into the chamber, a small entry hole (5 cm in radius) equipped with a short (15 cm length) netting sleeve on the outside and a flap aluminum gate with a spring mechanism on the inside of the exposure chamber is sealed on the front face of the exposure (or mosquito release) chamber. On the opposite face, a rear exit portal is composed of a horizontal opening, 10 cm long and 10 cm wide, at the end of an outward projecting funnel. A rectangular cube, 20 cm long and 10 cm wide, serving as an exit trap for collection of the 'escaped' mosquitoes with an aspirator at the end of each test, is attached over the exit funnel. On the distal side of this rectangle, like the entry hole, an orifice is cut which is equipped with a netting sleeve on the outside (Fig. 1). A cylindrical, 25-mesh wire screen, serving as an animal (guinea-pig) bait holder, 10 cm long and 7 cm diameter, is inserted onto the floor of the exposure chamber. Insecticide treated (test) or untreated (control) nets cover the bait holder accordingly. The whole E-R test system is run in complete darkness by covering in a black hood.

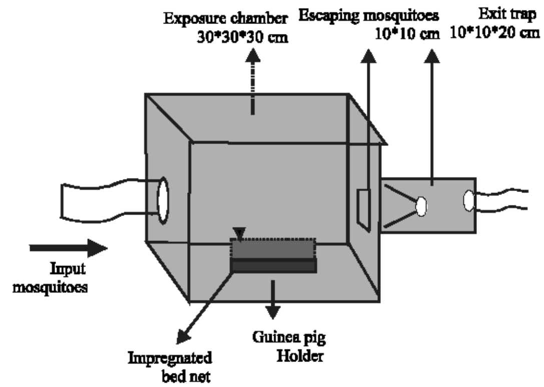

Fig. 1: An excito-repellency test box for the study of behavioral responses of mosquitoes to insecticides

Net impregnation: The net was 100% polyester, white in color and with a mesh size of 2 mm and 1 m^2 in area, which absorbed about 27 mL of water under wet conditions. Insecticide impregnation of nets was carried out by standard dipping procedures. The chemicals were first diluted in water and the nets were then immersed for 10 min in solutions and well shaken. The nets were then removed and hanged on a plastic rope under shadow till dry out. After 24 h, they were collected and stored for later use in black plastic bags in the fridge.

Nets were thus impregnated with 12.5, 25 and 50 mg a.i. m^{-2} of lambdacyhalothrin and similarly of deltamethrin and 40, 80 and 100 mg a.i. m^{-2} of cyfluthrin. The control nets were left untreated. Three insecticides were thus used in behavioural tests.

- Cyfluthrin 5% EW [Cyano-(4-fluoro-3phenoxyphenyl)-methyl-3-(2,2-dichloroethenyl)-2, 2-dimethylcyclopropanecarboxylate] (99.8% purity). This chemical was received from Bayer Environmental Science, USA.

- Deltamethrin 2.5% SC [(S)-a-cyano-3-phenoxybenzyl (IR)-*cis-trans*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate] (99.7% purity). This chemical was obtained from Aventis Environmental Science, USA.
- Lambdacyhalothrin 2.5% SC [1α(S), 3α(Z)-cyano(3-phenoxyphenyl)methyl 3-(2-chloro-3,3,3-trifluoro-1-propenyl)-2,2-dimethylcyclopropanecarboxylate] (99.8% purity). This chemical was received from Zeneca, USA.

Mosquito rearing: All life stages were reared in an environmentally controlled (temperature 25±3°C, 70±5% relative humidity) insectarium at TUMS. Following adult emergence, female mosquitoes were starved for 24 h before the start of the E-R tests. Adult mosquitoes were provided cotton pads soaked with 10% glucose solution from the day of emergence. They were maintained in a 30×30×30 cm screened cage.

Behavioural tests: Following the assembly of exposure chamber, a guinea pig was placed in the animal bait holder which was then inserted into the exposure chamber. The test method consisted of enclosing 25 female mosquitoes in a chamber containing animal bait covered with insecticide-treated or untreated (control) test nets. The exposure chamber had an exit portal for mosquitoes to escape to a receiving cage. A full test consisted of a pair of treatment chambers and a pair of control chambers.

The results of mosquitoes' behavior were recorded after 30 min as dead, survived, recovered, blood-fed and retrieved in the exit trap. At the end of each test, the animal bait was removed. Live and dead (or knocked-down) mosquitoes were collected with aspirator and forceps from exit and exposure chambers. They were transferred to paper cups covered with nets and containing cotton pads soaked with dilute glucose as food. The escaped mosquitoes and the remaining test specimens collected from the exposure chamber were held separately for observation and scoring of mortalities after 24 h holding periods.

Tests performed: Only *An. stephensi* females were used in excito-repellency tests. Each test was replicated at least six times. To fulfill the goals of this research, tests were performed to compare the three insecticides, concentrations of insecticides and survival versus recovery rates among mosquitoes.

Data analyses: Statistical analysis tests were carried out by one-way analysis of variance (ANOVA) with computation of the significance of differences in the outcome of various treatments. These were determined by using arcsine-transformed data (Y = Arc Sin vP) on the SPSS for windows 6.1 program.

RESULTS

Blood feeding and mortality of mosquitoes: The results obtained from E-R tests on bednets impregnated with three pyrethroid insecticides at relevant concentrations on *Anopheles stephensi*, India strain and their comparison with control mosquitoes are presented in Table 1. As indicated, lower blood feeding rates were observed in insecticide-treated trials than in untreated controls. Indeed, at given concentrations of different pyrethroid insecticides, the blood feeding rates of mosquitoes were on average about fourfold lower in treated than in untreated trials. The mean blood feeding rates of female mosquitoes were at least two and a half times less with deltamethrin than with lambdacyhalothrin. These differences were statistically significant ($p<0.01$) (Table 2). The values of variance ratio (F) between the blood feeding level means indicated that there was increasing significant differences between lambdacyhalothrin versus cyfluthrin and lambdacyhalothrin versus cyfluthrin and deltamethrin insecticides at 1% probability level.

Consistently, higher mortalities were recorded in treated than in control trials. The mortality rates were on average about 42-fold higher in treated than in control trials. There was almost no knockdown or mortality of control mosquitoes exposed to untreated net. Untreated

Table 1: The E-R test results of three pyrethroid ITNs against *An. stephensi* under laboratory conditions

Repeat	Mortality rate	Survival rate	Recovery rate	Feeding rate	Entry index	Mosquito number	Concentration	Insecticides
6	36.8	63.2	29.3	19.0	16.3	25	12.5	Lambda Cyhalothrin
6	39.5	60.4	38.0	15.0	18.2	25	25.0	
6	47.3	52.6	27.7	13.2	14.2	25	50.0	
6	65.0	34.9	14.1	9.1	17.9	25	12.5	Deltamethrin
6	69.2	30.1	14.1	5.3	11.2	25	25.0	
6	65.2	34.7	12.5	4.0	13.6	25	50.0	
6	32.3	67.7	51.0	9.3	19.7	25	40.0	Cyfluthrin
6	33.0	67.0	53.8	8.5	18.7	25	80.0	
6	30.4	69.5	49.4	5.5	27.8	25	100.0	
6	1.1	98.9	0.0	41.3	1.4	25		Control

Table 2: The ANOVA test results of the three pyrethroids (L = lambdacyhalothrin, D = deltamethrin, C= cyfluthrin) against *An. stephensi*

Variance ratio (F)	Mean squares	DF	Sum of squares	Source	Insecticides
77.50***	959.87	2	1919.75	Between levels	(Mortality rate)
	12.38	6	74.31	Within levels	L D C
44.62***	975.35	2	1950.71	Between levels	(Survival rate)
	13.07	6	78.43	Within levels	L D C
88.19***	1074.18	2	2148.37	Between levels	(Recovery rate)
	12.18	6	73.08	Within levels	L D C
11.95**	79.14	2	158.29	Between levels	(Feeding rate)
	6.62	6	39.71	Within levels	L D C
3.68*	49.69	2	99.38	Between levels	(Exit rate)
Not sig.	13.48	6	80.87	Within levels	L D C

*** = 0.1%, ** = 1%, and * = 5% probability levels

control mortality rate was only 1.1%. The mortality rate due to deltamethrin impregnation was 1.6 times that of lambdacyhalothrin and twice the mortality rate due to cyfluthrin impregnation. With 30 min exposure of susceptible (Beech) *An. stephensi*, the difference in killing effects between the three insecticides was highly significant ($p<0.001$), although the difference in mortality rates between lambdacyhalothrin and cyfluthrin versus deltamethrin was not significant.

Entry index: The entry indices of mosquitoes in the exit chamber also indicated that only 1.4% of control mosquitoes attempted to escape, while in treated trials on average 17.5% entered the exit trap, so there has been a 12.5-fold increase from control to treated trials (Fig. 2). The highest mean entry index (i.e., deterrence) was recorded for cyfluthrin (22%). Nevertheless, there was no significant evidence of a difference in entry indices between the three insecticides (Table 2).

Recovery and survival rates: Following 24 h post-exposure maintenance periods in paper cups, after female specimens had been removed from or escaped from exposure chamber, the recovery rates of previously knocked-down mosquitoes were analyzed. While this rate was zero in untreated controls, its mean at various concentrations of deltamethrin, lambdacyhalothrin and cyfluthrin were about 13.5, 32 and 51.5%, respectively. Thus, exposure of female mosquitoes to deltamethrin insecticide gave not only the largest mortality rate, but also the smallest recovery rate. In fact, the mean recovery rate due to deltamethrin impregnation was 3.8 and 2.4 times less effective than with cyfluthrin and lambdacyhalothrin impregnations, respectively (Fig. 3). These differences were statistically very significant ($p<0.001$).

The survival rate, or the rate at which hungry female mosquitoes remained alive despite being exposed to animal bait covered with insecticide-treated or untreated bednets, was near 100% in the control specimens. The

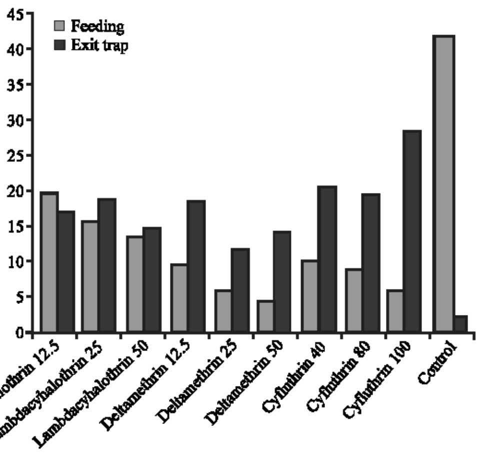

Fig. 2: Comparison of blood feeding rates and entry rates in to exit trap for *An. stephensi* in contact with the three pyrethroid ITNs

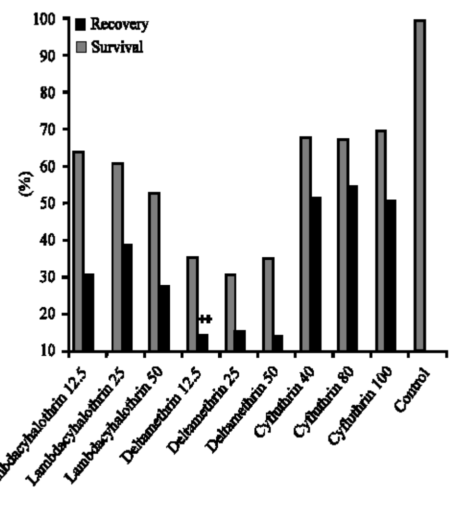

Fig. 3: Comparison of survival and recovery rates of *An. stephensi* in contact with the three pyrethroid ITNs

mean survival rate at various concentrations of different insecticides was about 53%. The mean survival rate of female mosquitoes exposed to cyfluthrin was twice that of deltamethrin. This difference was statistically very significant ($p<0.001$).

DISCUSSION

The international Roll Back Malaria partnership has advocated the use of insecticide-treated nets (ITNs) as one of the major preventive tools in the global control of malaria (RBM, 2003). One of the accessible aims of public use of treated nets is a reduction in mean age of the local mosquito population, so that fewer vector mosquitoes could harbor the malaria parasites. Comparing the three pyrethroid ITNs, the results of laboratory efficacy tests against the primary malaria vector mosquito, *An. stephensi*, in a baited Excito-repellency (E-R) exposure chamber indicated that deltamethrin was clearly most effective and cyfluthrin was least effective. These results confirmed the findings of earlier workers (Ansari *et al*., 1998).

On treated bednets, the pyrethroids act in three way: deterrence or escape due to the presence of some volatiles; irritance (or excito-repellence) due to a brief contact or minor exposure; and death due to an effective exposure to insecticide (Takken, 2002). Using one way analysis of variance (ANOVA), the results revealed that there was significant difference among pyrethroids ($p<0.05$) in the sequential effects of blood feeding, knockdown and mortality.

In a study in The Gambia by Lindsay *et al*. (1991), bednets impregnated with permethrin and lambdacyhalothrin reduced the blood feeding rate by 91% with respect to control. Another study, conducted by Thomson *et al*. (1995), has reported a statistically significant reduction in the human blood feeding index in one intervention trial. Studies of ITNs have generally yielded slight reductions in the blood feeding rates on humans which were not statistically significant (Lindsay *et al*., 1993; Mbogo *et al*., 1996; Magbity *et al*., 1997; Quinones *et al*., 1997). There was an estimated 25% reduction in blood feeding rates of *An. stephensi* mosquitoes on ITN-covered guinea pigs in the present study.

These results infer that deltamethrin is superior to other insecticides in terms of blood feeding. Compared with the other two insecticides, the highly marked values of blood feeding for mosquitoes under lambdacyhalothrin condition reflects that this pyrethroid has a lower contact irritability behavioral response. It has been demonstrated that blood feeding in females induces enzyme up-regulation, which may increase their resistance levels (Hemingway and Ranson, 2000; Holt *et al*., 2002). In addition, mating may also promote the female mosquito's ability to survive exposure to insecticides (Hunt *et al*., 2005).

The World Health Organization Pesticides Evaluation Scheme (WHOPES) has endorsed special compounds and dosages for their safety and efficacy when used for bednet treatment (Zaim *et al*., 2000). *An. stephensi* mosquito mortalities were somehow insensitive to whether the recommended dose of each pyrethroid insecticide or half or twice of that dose, had been applied to the netting. In a study by Hodjati and Curtis (1997) on this species, it was found that a higher dose of 500 mg m^{-2} of permethrin provoked more blood feeding and less knockdown and mortality compared with the lower dose of 200 mg m^{-2}, which was less irritating and more effectively insecticidal.

This discrepancy of low mortality at higher dose could be explained by the discernible earlier take-off of mosquitoes irritated by the higher dose of insecticide, resulting in shorter average exposure to the higher dose (Curtis *et al*., 1998). This reveals that mosquito mortality will continue at a high level even when washing or wear and tear has removed much of the insecticide amount recommended. Recently, Enayati *et al*. (2005) field-tested a novel recombinant glutathione S-transferase (GST)-based pyrethroid quantification assay for treated bednets.

Comparing the efficacy of the three pyrethroid insecticides, cyfluthrin was clearly least effective and deltamethrin was most effective in killing mosquitoes. These findings were consistent with those of earlier reports in which bioassay results on deltamethrin against vector mosquitoes showed significantly greater insecticidal power than alphacypermethrin and cyfluthrin (Adams *et al*., 2002).

The variation in proportions caught in exit traps indicates an excito-repellent effect of pyrethroid-treated nets, as found with nets impregnated with alphacypermethrin by Maxwell *et al*. (2003) and Soremekun *et al*. (2004). As indicated above, different insecticide concentrations appeared to have no clear effects on escape patterns in short-term exposure trials. Although there was no significant difference between the exit rates of the three pyrethroid insecticides, deltamethrin and cyfluthrin were on average the least and the most excito-repellent, respectively (Table 1). Other studies have also confirmed the least irritancy effect of deltamethrin on the malaria vector, *Anopheles* (Sharma *et al*., 2005; Vatandoost *et al*., 2005). It is important to note that the irritant properties of deltamethrin in ITN trials can have an unsatisfactory impact on malaria vector control activities. The E-R effect of deltamethrin may also culminate in a 'mass killing effect' on the village mosquito populations (Ilboudo-Sanogo *et al*., 2001; Sharma *et al*., 2005).

The recovery rates of previously knocked-down mosquitoes showed that deltamethrin had the smallest effects. This is a valuable parameter since many mosquitoes may have inherent capacities to evade definite death following temporary exposure to insecticides. Moreover, studies on the survival rates indicated that again deltamethrin had the least survival values. Survival rate is the reciprocal of mortality rate. Under laboratory conditions, many field-operational factors (such as mating) are changed which may affect the outcome of results. Attempts should be made to surmount such problems.

After a decade of laboratory maintenance, the *An. stephensi* Beech strain colony used here remained susceptible and responded well, to the three insecticides. In order to standardize behavioral responses of vector mosquitoes to different insecticides, field populations of malaria vectors should also be incorporated into such studies.

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