J Vect Borne Dis 40, March–June 2003, pp 54–59

Irritant effect, prevention of blood feeding and toxicity of nets impregnated with different pyrethroids on *An. stephensi*

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A resistant strain of *An. stephensi* was confirmed to have high resistance to knock down and kill by four different pyrethroids in bioassays in comparison with a susceptible strain. Permethrin, lambda-cyhalothrin and deltamethrin were more irritant to the susceptible strain than the resistant as judged by time for first take-off, but nets treated with alphacypermethrin appeared to be more irritant to the resistant *An. stephensi* than the susceptible. With all the pyrethroids tested, the resistant strain spent a longer time in contact with a treated net, which was in contact with a human arm, than did the susceptible strain. With permethrin the resistant strain fed significantly more successfully through the treated netting than did the susceptible strain. With deltamethrin there was a non-significant tendency in the same direction in comparing the two strains. However, with alphacypermethrin there was a non-significant tendency in the reverse direction. After 15 min in the cage which tested for the ability to feed through a pyrethroid treated net, observed mortality was higher with the susceptible than the resistant strain. Thus there was no sign that the longer resting of the resistant strain on treated netting would compensate for the fact that a higher dose was needed to kill this strain. Such compensation has been suggested with West African *An. gambiae* where treated nets continue to work well against a highly resistant wild population. However, this does not seem to apply to our resistant *An. stephensi.*

Key words *An. stephensi* – pyrethroid impregnated bednets

Synthetic pyrethroids and DDT have two types of toxic effect on insects—an initial rapid knock down effect, followed by a lethal effect. An excito-repellent effect is also shown by DDT and pyrethroids. This effect may be regarded as a disadvantage as it could drive away an insect before a lethal dose has been absorbed. However, there are beneficial features of this characteristic, because mosquitoes can be driven away from houses to bite animals, or to rest in less favourable outdoor sites and they may be prevented from biting through a treated net or from exploring a treated net for long enough to find a hole in it.

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In some cases DDT or pyrethroid resistant genes cause reduced irritability. For example, selection for DDT resistance in the spotted root maggot, *Euxesta notata,* resulted in decreased irritability[1,2]. Many DDT-resistant strains of mosquitoes showed less irritability than susceptible strains[3–5]. Hodjati and Curtis[6–8] reported the same for permethrin and pyrethroid resistant *An. stephensi.* Chandre *et al* [9] studied the irritant effect of 1% permethrin paper on *An. gambiae* in WHO bioassay cones. They reported that permethrin was more irritant to the susceptible strain than to the resistant mosquitoes carrying the *kdr* gene. Chandre *et al* [9] and Darriet *et al*[10] concluded that

pyrethroid impregnated bednets in laboratory and experimental hut tests provided good levels of protection against biting and high mortality of *kdr* resistant strains of *An. gambiae.*

The protection in experimental huts was at least as good when the *kdr* frequency was 94% as when it was lower[10]. Furthermore in a high *kdr* area lambdacyhalothrin treated nets protected well against malaria and lowered the density and sporozoite rate of the vector population[11]. It was suggested that all these surprising effects were because of the reduced irritability due to *kdr* caused mosquitoes to rest longer on a treated net and eventually to pick up a lethal dose. If this can be confirmed as a general phenomenon it would have the very important implication that the emergence of at least some forms of resistance would not interfere with effective use of treated nets contrary to earlier fears that such resistance would be a fatal blow to the WHO's strategy for malaria prevention. Therefore, the present study investigated in the laboratory the insecticidal, irritant and blood feeding prevention effects of nets treated with various pyrethroids against a susceptible and a highly pyrethroid resistant strain of *An. stephensi.*

Material & Methods

Mosquitoes and netting

Three to five days old glucose fed female mosquitoes of the following strains were used in this study:

Anopheles stephensi (BEECH): susceptible to permethrin and other insecticides; originally from India and maintained at London School of Hygiene and Tropical Medicine (LSHTM) for many years.

Anopheles stephensi (DUB234): originally collected as larvae from the field in Dubai (United Arab Emirates) and colonised at the Liverpool School of Tropical Medicine as the DUB-S strains. Larvae and adults showed a high level of resistance to DDT and some resistance to permethrin. Adults and larvae were subjected to selection for permethrin resistance[12]. Eight generations of selection on the adults increased the LT_{50} by seven fold compared to the parental DUB-S stock, and by 10 fold compared to an Indian stock of the same species. The selected Dubai strain has been kept under selection pressure since 1989 at LSHTM by exposing adult mosquitoes to 0.25% permethrin for 1 h until 1994 and then the exposure time was raised—newly emerged male and female mosquitoes of successive generations were exposed to permethrin for 2, 3, 4 and eventually to 5 h and the surviving mosquitoes were used to produce the next generation. At the end of the selection procedure, the selected mosquitoes of DUB234 had 32-fold resistance to permethrin compared to the susceptible BEECH strain. Pyrethroid resistance in the Dubai strain appears to include both reduced sensitivity of the active target site—a *kdr*-type mechanism and metabolic mechanisms[13–15].

Netting, insecticides and impregnation

The netting used in this study was 100% polyester, white in colour and with a mesh size of 1.5 mm, made by the Siam Dutch Netting Co., Bangkok, Thailand. The insecticides used were:

Permethrin (25% cis : 75% trans, 10% E.C.), Roussel Uclaf (now Agr Evo), UK; Permethrin (25% E.C.), Coopex; Alphacypermethrin 'Fastac' (10% B.C.), Cyanamid of Great Britain Ltd.; Alphacypermethrin 'Fendona' (10% E.C.); BASF; Lambdacyhalothrin 'Icon' (2.5% E.C.), Zeneca, UK; and Deltamethrin (5% S.C.), Vapco, Jordan.

Pieces of polyester net with known area were impregnated with 500 or 200 mg/m^2 permethrin, 20 mg/m^2 alphacypermethrin, 25 mg/m^2 deltamethrin or 25 mg/m^2 lambdacyhalothrin, taking into account the uptake of liquid after dipping and wringing a known area of net. Each piece of net was labelled and soaked for 5 min in the appropriately diluted emulsion and then excess liquid was wrung out. After treatment the nets were hung in a well ventilated room for 48 h.

Testing methods

Tests of knock down and mortality of the susceptible and resistant strains: The knock down effect of nets impregnated with permethrin, alphacypermethrin or deltamethrin was studied with the nets wrapped round a spherical wire frame so that the mosquitoes could not escape contact with the netting.The times for first knock down of the first, median and last mosquitoes of the susceptible and resistant strains were observed when they were left continuously exposed on the netting. The knocked down mosquitoes were kept in a paper cup and provided with a glucose soaked cotton and the final mortality was recorded after 24 h holding. Five replicates with 11 mosquitoes were carried out for each strain and with each insecticide concentration. For each treatment and strain 2–3 replicates of 11 mosquitoes each were tested as controls.

To test the effectiveness of the resistance in a set-up more similar to the bioassay recommended by the WHO[16] batches of 15 four to five day old glucose fed females of each strain were exposed for 3 min to the permethrin, alphacypermethrin and deltamethrin impregnated netting wrapped round the above described spherical wire frame. Exposed mosquitoes were transferred to paper cups and provided with a pad of glucose solution. Mortality was recorded 24 h after the end of the exposure period. The number of replicates for each strain were 5, representing 75 mosquitoes of each strain for each treatment. For each treatment and strain 2 replicates of 15 mosquitoes each were tested as controls.

Irritability tests

To compare the irritant effect of nets impregnated with different pyrethroid insecticides to the susceptible and resistant mosquitoes these were individually exposed to the nets either (a) in a WHO plastic cone pinned to the vertical side of a polystyrene box or (b) inside a spherical frame made by soldering together two intersecting circles of wire (12 cm in diameter) which were completely covered with a piece of net to be tested or, (c) inside a mosquito cage measuring 25 x 25 x 25 cm where mosquitoes were offered the opportunity to feed blood through the top face of the cage which had been pyrethroid treated.

One female mosquito was gently introduced into the sphere or bioassay cone by means of a mouth aspirator and a stop watch was started as soon as the mosquito landed on the netting. The time taken before the first take-off was recorded with or without allowing a 1 min settling period in which observations were not recorded. The use of such a settling period was recommended for studies with DDT[17].

To test the irritant effect and prevention of blood feeding of a net, one glucose fed female mosquito was gently introduced into the cage described above by means of a mouth aspirator and the experimenter placed one of his arms on the top of the cage over the treated netting. As soon as the mosquito landed on the treated netting and started feeding a stop watch was started. The time taken before the first take-off was recorded. The time the mosquito spent on the treated netting before each take-off during an observation period of 15 min was observed and used to calculate the accumulated time spent on the treated netting by the mosquitoes. Time spent resting on the untreated side of the cage was excluded from this count.

Before starting the test, the readiness of the mosquitoes for blood feeding was examined by providing them a bared arm over the rearing cage for 15 sec when the test started, if the mosquito did not start feeding in 3 min it was excluded from the test.

The number of knocked down mosquitoes was recorded and, when the observation time ended, the mosquitoes were removed from the cage by means of a mouth aspirator and placed in a paper cup with a pad of glucose soaked cotton provided on the top of the gauze covering. The cup was kept in the laboratory at 70–80% humidity and after 24 h the final mortality was recorded. All tests were carried out at 28–

30°C in a laboratory with artificial light coming from the ceiling and after a minimum of 60 min adaptation of the mosquitoes to the conditions in the testing laboratory.

Twenty replicates of tests were carried out for each strain and treatment. Four to seven replicates of control tests were carried out in each case. All statistical analyses were done using the SPSS for the Windows 11.01 program. Significance tests between two means were done by 't' test. The significance of the difference in the final mortality for different treatments was assessed by chi-square tests.

Results & Discussion

The results in Table 1 show that the median time for knock down was significantly higher for the resistant than the susceptible strain with all three of the pyrethroids tested. There was no knock down in the control mosquitoes.

Bioassay tests showed no mortality with the control netting and high mortality of the susceptible strain with all the three pyrethroids but low mortality of the resistant strain (Table 1). Thus the resistant strain is unequivocally resistant to the knock down and insecticidal effects of three different pyrethroids.

The results in Table 2 show that with permethrin and lambdacyhalothrin the resistant mosquitoes were significantly less irritable (as measured by time for first take-off) than the susceptibles. The apparent difference in the opposite direction between the two strains with alphacypermethrin was not significant. The con-

Table 1. Time (sec) for median knock down with continuous exposure and per cent mortality after 3 min exposure of pyrethroid susceptible (BEECH) or resistant (DUB234) *An. stephensi* in contact with pyrethroid treated netting in a wire frame wrapped with the treated netting

Test method	Permethrin[c] (200 mg/m^2)			Alphacypermethrin[f] (20 mg/m^2)			Deltamethrin (25 mg/m^2)		
	Susceptible		Resistant	Susceptible		Resistant	Susceptible		Resistant
Median knock down time (sec)	502	< * >	1776	566	< *>	1425	655	< * >	1963
Per cent mortality after 24 h holding of the knocked down mosquitoes	91%	< *** >	9.1%	100%	< *** >	69%	91%	< *** >	7.3%
Per cent mortality of 3 min exposure to the treated nets and after 24 h holding	67%	< *** >	1.3%	100%	< *** >	6.7%	65%	< *** >	2.7%

[c]Coopex; [f]Fendona; Significance of the differences between strains is shown by *p<0.05; **p<0.001; ***p<0.001.

Table 2. Time (sec) for the first take-off of pyrethroid susceptible (BEECH) or resistant (DUB234) *An. stephensi* in contact with pyrethroid treated netting

Test method	Permethrin[r] (500 mg/m^2)			Alphacypermethrin[f] (20 mg/m^2)			Lambdacyhalothrin (25 mg/m^2)		
	Susceptible		Resistant	Susceptible		Resistant	Susceptible		Resistant
In WHO cone (with 90 sec settling period)	16	< *** >	60	55	< ns >	28	–		–
In wire frame wrapped with netting (no settling period)	15	< *** >	219	–		–	129	< *** >	412

[r]Roussel Uclaf (Agr Evo) product; [f]Fastac Indicators of significance as in Table 1; ns — non-significant.

trol mosquitoes generally landed on the untreated control netting and remained on it for the entire observation time, rarely leaving it.

The irritability results in this study for mosquitoes of susceptible and resistant strains of *An. stephensi* to 500 mg permethrin/m^2 are consistent with the results presented in earlier studies by Hodjati and Curtis[6–8].

Table 3 shows results in a cage when mosquitoes had access to a blood meal through treated nets. With permethrin there was a non-significant tendency for a longer time for first take-off with the resistant than with the susceptible strain. With alphacypermethrin there was a non-significant tendency in the reverse direction.

These results resemble the difference between these two pyrethroids seen in Table 2. With deltamethrin there was a significantly delayed first take-off in the resistant strain compared with the susceptible, as seen with lambdacyhalothrin in Table 2. With all three pyrethroids tested, the resistant strain spent a longer time in contact with the treated net and the human arm than did the susceptible strain.

With permethrin the resistant strain fed significantly more successfully through the treated netting than did the susceptible strain. With deltamethrin there was a non-significant tendency in the same direction in comparing the two strains. However, with alphacypermethrin there was a non-significant tendency in the reverse direction.

There was a high mortality due to permethrin and alphacypermethrin with the susceptible strain, but the resistance protected significantly against these effects. With deltamethrin there was only 20% mortality with the susceptible strain and none with the resistant.

All mosquitoes of the susceptible and resistant strains tested on an untreated cage fed during the observation period. There was no knock down or mortality after 24 h and they spent almost all the 15 min observation period on the face of the net to which the human arm was applied.

We were hoping to confirm that, with a pyrethroid resistant strain, reduced irritability in pyrethroid treated netting would lead to longer resting on the treated surface so that eventually a lethal dose was picked up, thus explaining the surprisingly good results with treated bednets against a resistant West African wild population[10,18].

Our results confirmed longer accumulated time resting on the treated surface with three pyrethroids (Table 3), even with alphacypermethrin which showed

Table 3. Measures of irritability, prevention of blood feeding and insecticidal power with susceptible (BEECH) and resistant (DUB234) *An. stephensi* when offered the opportunity lasting 15 min to blood feed through one face of a netting cage which had been pyrethyroid

Test method	Permethrin[c] (200 mg/m^2)			Alphacypermethrin[f] (20 mg/m^2)			Deltamethrin (25 mg/m^2)		
	Susceptible		Resistant	Susceptible		Resistant	Susceptible		Resistant
Time (sec) for first take-off	108	< ns >	148	91	< ns >	50	190	< ** >	404
Mean accumulated time on treated net and human arm	184	< ** >	349	150	< *** >	323	318	< ** >	538
Per cent blood fed through treated netting	30%	< *** >	85%	40%	< ns >	25%	70%	< ns >	95%
Per cent knocked down	80%	< *** >	0	20%	< ns >	0	0		0
Per cent mortality after 24 h holding	85%	< *** >	5%	60%	< ** >	15%	20	< ns >	0

[c]Coopex; [f]Fendona; Indicators of significance as in Table 1; ns — non-significant.

no evidence for less irritability with the resistant strain (Table 1). However, during the long periods standing on the treated netting the resistant mosquitoes fed as much as, or more than the susceptible. Eventual mortality was less with the resistant strain than the susceptible and this difference was highly significant for two of the pyrethroids.

Thus our results do not conform with the reassuring idea that reduced irritability would compensate for the higher doses needed to kill resistant strains. Our results suggest that resistance of the type found in the Dubai *An. stephensi* stock would interfere very seriously with the functioning of pyrethroid treated nets and would prevent their providing personal protection to the net user and a community-wide impact on the infective biting population.

Acknowledgement

M.H. Hodjati and N. Mousavi received financial support from the Iranian Ministry of Health and Tabriz University of Medical Sciences during their sabbatical leaves at LSHTM. C. Curtis has a programme grant from the British Medical Research Council for research on treated nets against malaria mosquitoes.

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