Detection of pyrethroid resistance in *Anopheles* mosquitos

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Although pyrethroid insecticides are a promising means of controlling Anopheles *malaria vectors, there is a need to monitor for resistance. It has been proposed that the results of the WHO-recommended testing method, involving exposure to impregnated paper for 1 hour, might be misleading because of knockdown during this period, and that exposure to a higher dose of pyrethroid for 2 minutes might be preferable. However, comparative tests with a susceptible and a permethrin-resistant strain of* A. stephensi *showed that exposure for 1 hour was at least as sensitive in detecting resistance as was the short exposure method.*

Introduction

Pyrethroid insecticides impregnated into bednets[a] or used for conventional residual house-spraying (*1*) are a promising weapon against malaria mosquitos, the rapid knockdown effect being an important advantage. However, there is concern that pyrethroid resistance may be selected in *Anopheles* mosquitos as a result of either antimalarial or agricultural applications.

For several years, WHO has made permethrin- and deltamethrin-impregnated papers available for use in standard susceptibility test kits. The diagnostic dosage recommended for permethrin has been 0.25% (w/v) with exposure lasting 1 hour (*2*). In 1992, the WHO Expert Committee on Vector Biology and Control recommended various diagnostic dosages of permethrin for different *Anopheles* species, but exposure for 1 hour continued to be recommended for all except one species (*3*). However, it has long been recognized that the quick knockdown caused by pyrethroids may give equivocal results (*4*). Susceptible mosquitos are not expected to spend the intended 1 hour absorbing insecticide through their tarsi: with a vertical exposure tube they would be quickly knocked down and fall on to the plastic base of the kit, where they might recover before the end of the 24-hour holding period and be scored as survivors. Conversely, if the tube were kept horizontal and relatively tolerant mosquitos were knocked down they would lie on the impregnated paper and could be expected to absorb a large, possibly lethal, dose. Because of these problems, the Expert Committee recommended further studies on methods of testing for pyrethroid resistance (*3*). We report here the results we obtained in 1988 and 1994.

Method

Our work was based on the following considerations and expectations.

- Tests are likely to be more meaningful if operationally realistic dosages and substrates are employed. We therefore used nylon mosquito netting impregnated with permethrin according to the method described by Hossain et al. (*5*) at a range of dosages similar to those used on bednets. The netting was used as a lining, backed by a paper stiffener, in WHO test kits.
- Exposure should only be for 2 minutes so that no mosquitos are knocked down until after the exposure period, and all therefore have the same chance of absorbing insecticide through their tarsi. Such a short exposure also seems likely to give a more meaningful test since pyrethroids are irritants and mosquitos do not spend long periods resting on bednets that are impregnated with permethrin (*6*).
- Possible diagnostic dosages and exposure times should be evaluated, not merely for their ability to kill susceptible insects reliably, but also to discrimi-

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nate resistant from susceptible strains. An *A. stephensi* strain of Dubai origin, selected at the adult stage in the laboratory with permethrin by Laddoni & Townson (*7*), and designated Dub/Apr, provides one of the few clear-cut cases of pyrethroid-resistant *Anopheles*.

Results and discussion

In 1988 we compared the Dub/Apr resistant strain with the Beech susceptible strain of the same species using the testing system described above and also the conventional WHO system using paper impregnated with 0.25% permethrin (equivalent to 97 mg/m^2) and a 1-hour exposure. In each case the tests were conducted at 25 °C and the mosquitos were held for 24 hours after exposure before mortality was recorded.

Exposure for 2 minutes to 200 mg permethrin/m^2 of netting almost completely discriminated between the two strains, but a few of the Beech strain survived and a few of the Dub/Apr strain were killed by the next higher dose tested (Table 1). The LD_{50} values calculated from the results for the range of doses tested indicated a resistance ratio of 370:1.

Contrary to expectation, the standard WHO test was better at discriminating between the two strains: at exposures of 1–2 hours there was a 100% kill of the Beech strain, whether the exposure tubes were held vertically or horizontally, while none of the Dub/Apr strain was killed (Table 2). Only when the exposure time was increased to 4 hours did a few specimens of the latter strain die.

We repeated the tests in 1994, by which time the Dub/Apr strain was considerably less resistant than it had been in 1988. On netting, none of the doses came near to discriminating the strains. The tests with 0.25% permethrin papers and with 0.1% lambda-cyhalothrin papers gave the results shown in Table 2. Exposure for 1–2 hours permitted considerable, but nevertheless incomplete, discrimination to be made between the two stocks for each of the pyrethroids. There was little difference in the results with the tubes in the vertical or horizontal positions.

Unexpected results in resistance tests are commonly ascribed to the use of old test papers. In an effort to quantify the effect of repeated use, we exposed successive batches of the Beech stock to the same paper, keeping it refrigerated between tests. With a 1-hour exposure in the horizontal position, mortality was 100% until the seventeenth use of the same paper, by which stage a total of 255 mosquitos had been exposed. The mortality dropped to 50–90% for the next 50 mosquitos exposed, even though the WHO-recommended expiry date of the paper had not been reached. We therefore suggest that a test paper should not be used to expose more than a total of 150 mosquitos.

Table 1: **Percentages of Beech (susceptible) and Dub/Apr (resistant) strains of *Anopheles stephensi* knocked down 1 hour after and dead 24 hours after exposure for 2 minutes to various doses of permethrin on nylon netting**

Dose (mg/m^2)	Beech strain			Dub/Apr strain		
	% knocked down	% dead	No.[a]	% knocked down	% dead	No.[a]
25	20.0	21.2	85	0	0	106
50	80.4	52.2	92	0	0	102
100	96.1	83.3	78	0	0	100
200	91.6	97.6	83	0	0	104
400	100.0	100.0	77	3.4	1.7	118
800	—	—	0	17.3	3.8	104
1 600	—	—	0	79.0	10.0	100
2 500	—	—	0	88.4	12.5	104
3 200	—	—	0	67.0	17.5	97
LD_{50}(mg/m^2)	47			17 400		

[a] No. of mosquitos tested.

The results shown in Table 2 have increased our confidence that the WHO-recommended test system is likely to identify cases of pyrethroid resistance. It is being used with 0.025% (w/v) deltamethrin papers by the Antiparasitic Diseases Institute, Sichuan Province, China, to test for resistance in an area where 2.25 million bednets have been impregnated with deltamethrin (*8, 9*). However, we also advocate testing under conditions that are as realistic as possible, including those where the mosquitos are free-flying but are stimulated to land on an impregnated net (*10*). Pyrethroid-resistance genes of various levels of protective efficacy can be expected to arise; it is important to detect them at an early stage and to assess their likely impact on control operations.

Note added in proof. It has recently been found that better discrimination can be obtained by observing the time for knockdown of mosquitos left confined in a WHO bioassay core in contact with an impregnated net—all of the Beech stock are knocked down several minutes before the first of the Dub/Apr stock.

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Table 2: **Proportions of the Beech and Dub/Apr strains of *Anopheles stephensi* knocked down at the end of various exposure periods to papers impregnated with 0.25% permethrin or 0.1% lambda-cyhalothrin and the proportions dead after 24 hours**

Exposure time (hours)	Beech strain						Dub/Apr strain					
	Tubes vertical			Tubes horizontal			Tubes vertical			Tubes horizontal		
	% knocked down	% dead	No.[a]	% knocked down	% dead	No.[a]	% knocked down	% dead	No.[a]	% knocked down	% dead	No.[a]
Permethrin:												
1988 results												
0.25	6.5	71.4	105	6.2	50.0	128	0	0	72	—	—	0
0.5	59.2	79.9	189	68.0	75.0	172	0	0	69	—	—	0
1	98.1	100.0	109	100.0	100.0	124	0	0	74	—	—	0
2	100.0	100.0	100	100.0	100.0	119	0	0	76	—	—	0
4	—	—	0	—	—	0	8.6	12.3	105	—	—	0
6	—	—	0	—	—	0	35.4	45.5	79	—	—	0
8	—	—	0	—	—	0	47.4	61.8	76	—	—	0
1994 results												
0.25	19.0	48.0	100	26.6	52.0	75	10.0	10.0	100	0	15.3	130
0.5	44.4	60.0	90	41.2	66.2	80	10.0	10.0	100	0	2.0	100
1	85.8	77.5	120	81.9	80.1	111	14.4	9.6	125	0	5.1	135
2	98.1	99.0	110	97.1	98.5	140	36.9	50.7	130	32.8	47.2	125
4	100.0	100.0	90	100.0	100.0	90	36.3	41.8	110	38.0	40.9	105
6	—	—	0	—	—	0	37.0	35.1	108	76.1	72.3	105
8	—	—	0	—	—	0	97.5	100.0	40	95.0	95.0	45
Lambda-cyhalothrin:												
1994 results												
0.25	52.5	90.8	120	50.8	94.0	118	12.5	29.1	120	7.2	12.0	125
0.5	97.7	98.8	87	76.0	96.0	75	26.3	21.0	95	35.7	24.2	70
1	93.6	92.7	110	98.0	96.1	105	40.0	25.6	125	43.7	46.8	160
2	97.6	98.8	85	100.0	100.0	80	86.0	43.3	150	85.1	40.7	135
4	98.5	100.0	70	100.0	100.0	72	98.1	94.5	110	98.3	81.6	120
6	—	—	0	—	—	0	94.0	90.0	50	90.0	86.0	50
8	—	—	0	—	—	0	100.0	100.0	40	100.0	100.0	40

[a] No. of mosquitos tested.

Résumé

Détection de la résistance aux pyréthrinoïdes chez les anophèles

Les insecticides à base de pyréthrinoïdes constituent un moyen de lutte prometteur contre les anophèles vecteurs du paludisme. On peut en imprégner les moustiquaires ou les vaporiser de façon conventionnelle. Comme ces substances sont de plus en plus employées, il est nécessaire de surveiller la résistance aux pyréthrinoïdes. Les pyréthrinoïdes «assomment» rapidement les insectes et l'on a pensé que l'épreuve de sensibilité recommandée par l'OMS, qui expose les moustiques pendant une heure à du papier imprégné avec une solution de perméthrine à 0,25% (ce qui équivaut à 97 mg/m^2), pourrait être une source d'erreur à cause de cet effet de *knock-down* pendant la période d'exposition. A l'aide d'une souche sensible d'*A. stephensi*, et d'une souche de la même espèce originaire de Doubaï, ayant été choisie pour sa résistance à la perméthrine, nous avons comparé la méthode classique, avec des durées d'exposition allant de 15 min à 8 heures, à une exposition pendant 2 min à une moustiquaire imprégnée de perméthrine (25 à 3200 mg/m^2). Contrairement aux résultats attendus, il s'est avéré que la méthode classique était au moins aussi sensible pour déceler la résistance que l'exposition pendant 2 min. On applique donc la première en Chine, où les moustiquaires imprégnées sont utilisées à grande échelle, pour surveiller la résistance. Si l'on décèle un cas de résistance, il est recommandé d'effectuer des épreuves sur des moustiques volant librement,

afin d'évaluer la probabilité que cette résistance ait un impact sur les opérations de lutte.

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