A SIMPLE METHOD OF IDENTIFYING ORGANOPHOSPHATE INSECTICIDE RESISTANCE IN ADULTS OF THE YELLOW FEVER MOSQUITO, *AEDES AEGYPTI*

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ABSTRACT. A simple filter paper spot test is described for identifying adult *Aedes aegypti* resistant to malathion as a result of raised esterase levels. The method is compared with established polyacrylamide gel (PAG) electrophoresis techniques for determining esterase-6 activity, and its applicability as a field test is discussed.

INTRODUCTION

The association between organophosphate (OP) insecticide resistance in mosquitoes and raised levels of esterase activity is now well established in 4 species of the *Culex pipiens* group (Yasutomi 1970, Pasteur 1977[2], Georghiou and Pasteur 1978, Curtis and Pasteur 1981, Villani et al. 1983), *Culex tarsalis* Coq. (Georghiou and Pasteur 1978.), *Culex tritaeniorhynchus* Giles (Yasutomi 1971) and *Anopheles culicifacies* Giles (Herath and Davidson 1981). Recent studies on OP-resistant *Aedes aegypti* (Linn.) larvae (Yasutomi 1983) and adults (Field et al. 1984) have demonstrated a similar association, the latter study using PAG electrophoresis and scanning densitometry to quantify the relationship.

However, all these studies have involved complicated laboratory-based methodologies such as the visualization of esterase bands by the techniques of electrophoresis and colorimetric analyses with or without the use of various synergists. This restricts their usefulness in the field as they necessitate the availability of trained staff working with fairly sophisticated laboratory equipment and requiring a considerable expenditure of time and other resources.

A simple filter paper spot test for assaying esterase activity was developed by Ozaki (1969) for studying OP-resistance in the leafhoppers *Nephotettix cincticeps* and *Laodelphax striatellus*. It was subsequently modified for analysis of field populations of *Culex pipiens* Linn. mosquitoes (Pasteur and Georghiou 1980, 1981). We have modified the technique and compared the results for adult *Ae. aegypti* with an established electrophoretic technique for visualisation and quantification of esterases.

The results obtained should establish whether such a spot-testing methodology would be useful in the detection of malathion resistance in field populations of *Ae. aegypti*.

MATERIALS AND METHODS

MOSQUITOES. The following 4 strains of *Ae. aegypti* were used:—

(a) *Villa Palmeras* (VP). Strain obtained from Puerto Rico in 1978. Exposed in the laboratory to strong malathion selection pressure on adults and larvae for each of 12 successive generations.

(b) *Unselected Villa Palmeras* (UVP). Strain as in (a) above, but maintained for 12 generations without exposure to malathion.

(c) *Triple Marker* (TM). Strain susceptible to malathion and homozygous for visible markers red (*re*), spot (*s*), yellow (*y*) and black-tarsi (*blt*), origin unknown but obtained from the University of Notre Dame, Indiana, USA.

(d) *National Institutes of Health Strain* (NIH). Origin unknown but maintained since 1930 at the National Institutes of Health, Bethesda, Maryland, USA. Selected to be homozygous for an *Est-6* band with a designated Rf value of 1.00.

Reciprocal mass-mated crosses were carried out between susceptible and resistant strains (i.e., TM × VP). Methods of rearing larvae and adults were as described by Hitchen (1972)[3]. LT 50 values were determined by the use of standard World Health Organization susceptibility tests, (W.H.O. 1963), using 6 different exposure times, 4 replicates of each and 25 individuals per test.

ANALYSIS OF ESTERASE ACTIVITY

(a) *Filter paper spot method using crude homogenate.* Initially, a filter paper spot test technique was devised based on that of Pasteur and Georghiou (1981) using individual mosquitoes which were crudely homogenized by placing each on a white tile in a drop of distilled water and

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[2] Pasteur, N. 1977. Recherches de genetique chez *Culex pipiens pipiens* L. Polymorphisme enzymatique, autogenese et resistance aux insecticides organophosphores. These de Doctorat d'Etat. Universite de Montpelier II.162 pp., cited in Pasteur and Georghiou 1980.

[3] Hitchen, J. M. 1972. Genetical studies on insecticide resistance in the mosquito *Aedes aegypti*. Ph.D. thesis, University of Manchester, 152 pp.

crushing the mosquito with the blunt end of a glass rod. This homogenate was then spotted onto filter paper and dried and the paper stained for esterases by immersion in a mixture of:

100 ml	0.1 M Phosphate buffer (pH 6.5)
0.02 g	α-naphthyl acetate in minimum volume of acetone
300 mg	Fast red TR

The papers were left in this mixture for 10 min, then fixed in 7% acetic acid in water and air-dried at room temperature. The papers were then examined visually to see if there were any obvious and consistent differences between strains.

(b) *Comparative investigation of filter paper spots and electrophoretic bands.* Three day old adults of each strain were sexed and weighed. Each individual was separately homogenized in 25 μl of distilled water and the homogenate collected in microhaematocrit capillaries. These were subsequently centrifuged at 12,000 G for 3 min. The supernatant was then used for both methods as follows:

(1) Electrophoresis. Five μl aliquots from each supernatant were transferred to separate wells in a 7% polyacrylamide gel prepared as described in Field et al. (1984). Each gel was produced so as to contain at least one reference sample of each of VP, TM and NIH strains. The NIH sample was used as a standard to confirm the band mobilities and to enable the identification of the esterases relative to those of Field et al. (1984) All samples on any one gel were of the same sex. Following electrophoresis, the gels were stained for esterases using the methodology of Field et al. (1984).

(2) Filter paper spot method. Twenty μl aliquots from each supernatant were applied to appropriately labelled 16 × 8 cm pieces of Whatman No. 2 filter paper previously marked into 2 × 2 cm squares. These were treated and stained as in (a) above.

The papers were initially examined visually to see if there was any obvious and consistent difference between resistance phenotypes (strains) and crosses. Intensity levels of Esterase-6 band(s) (Field and Hitchen 1981), total esterase bands and spot intensity were measured on an Hellena Quickscan Jr. densitometer fitted with a green filter (525 nm). The area under the curve(s) for each specimen was then calculated and the figures obtained converted to $\log_{10}$ to normalize the distributions. The mean values for each sex and strain were compared by a one-way analysis of variance and Scheffe's S-test (Scheffe 1959).

These combined procedures permitted the comparison of esterase activity as measured by the spot test between strains and crosses and they also allowed for a correlation of esterase levels measured by spot test and PAG electrophoresis for each individual mosquito to be calculated.

RESULTS AND DISCUSSION

Visual examination of stained spots prepared without centrifugation revealed a clear and consistent difference between the intensity of TM spots and those of all other types, in that the TM was markedly less intense. Similar results were obtained with centrifuged material.

The weights of individual males and females in each of the strains investigated, and the LT_{50} values are shown in Table 1, together with the mean $\log_{10}$ esterase intensities as measured by the spot-test method for each of the strains and the 2 reciprocal crosses. A one-way analysis of variance conducted on the mean $\log_{10}$ esterase intensity data in Table 1 gave a variance ratio $F = 130.25$, $p<0.001$. Paired comparisons of the various treatment groups using Scheffe's S-test on pooled and ungrouped data are given in Table 2, together with the inferences from the results. This allows a comparison to be made between these results and those for esterase band intensities in Field et al. (1984), the use of the NIH standard marker ensuring that the bands identified here were the same as those in his study.

The relationship between $\log_{10}$ total esterase activity measured by the spot method, and $\log_{10}$ esterase-6 activity as measured by PAG electrophoresis for 10 randomly chosen individuals of each sex of each strain (except for TM for which there are 12 points, thus allowing a calculated r value with 100 degrees of freedom) is shown graphically in Fig. 1. The regression line for the scatter diagram is highly significant ($r = 0.5318$, $p_{100\ df} << 0.001$).

The line demonstrates that most of the esterase activity is that of esterase-6, and it is this which is responsible for the variation in total esterase between resistant and susceptible strains. The contribution of esterase-6 to malathion resistance in *Ae. aegypti* is discussed in Field et al. (1984).

The present study demonstrates that using crude preparations of adult *Ae. aegypti*, it is possible to carry out a simple filter paper spotting test which will differentiate between a susceptible strain and strains which are resistant to malathion as a result of raised esterase levels. The study also enables these results to be compared to those obtained by using the established laboratory techniques for visualization of esterase bands on electrophoretic gels. The

Table 1. Data used to compare $\log_{10}$ total Esterase intensity as measured by spot test in adult *Aedes aegypti*.

Strain[a]	Treatment group	Sex	Mean body weight (mg ± SE)[b]	LT_{50} (min)	No. of observations per treatment	Mean $\log_{10}$ Esterase intensity	Standard deviation
TM	1	Female	2.27 ± 0.03	4.2	270	2.684	0.253
	2	Male	0.97 ± 0.02	2.4	270	2.562	0.318
VP	3	Female	2.52 ± 0.03	39.5	270	3.174	0.239
	4	Male	0.98 ± 0.02	24.4	268	2.867	0.309
F_1 (TM × VP)	5	Female	2.64 ± 0.03	28.4	105	3.132	0.259
	6	Male	1.35 ± .0.01	14.6	1.05	2.963	0.266
F_1 (VP × TM)	7	Female	2.50 ± 0.02	21.6	105	3.209	0.242
	8	Male	1.31 ± 0.01	13.2	105	3.052	0.120
UVP	9	Female	2.48 ± 0.02	14.4	110	3.319	0.195
	10	Male	1.12 ± 0.02	9.7	110	2.978	0.282

[a] Females written first in crosses.

[b] Spearman rank-order correlation coefficient for mean body weight and mean $\log_{10}$ esterase intensity: females $r = 0.04$, $p > 0.10$; males $r = 0.71$, $p > 0.10$.

analysis of total esterases using the spot method does not enable differentiation between resistance phenotypes as was obtained in the study by Field et al. (1984) where electrophoretic bands of Esterase-6 were scanned.

The results obtained for quantification of spot intensity by scanning (Table 2) indicate that while it is possible to differentiate between TM and all other strains and crosses, it is not possible to distinguish resistance phenotypes, i.e., in this instance simple visual examination of crude filter paper spots provides as much information as the more sophisticated laboratory methodology.

The lack of a significant correlation between body weight and $\log_{10}$ total esterase activity for both males and females is in agreement with previous results (Field et al. 1984). This is despite the fact that the malathion susceptible TM strain was lighter in weight than any of the other treatment groups of the same sex. Any differences observed in the levels of esterase activity should therefore be due to differences between the strains other than variation in the sizes of the organisms. However, it is possible that the slight variation in weight between the strains may be an additional mechanism of resistance, since there is evidence that probit-mortality for DDT and malathion may be linearly related to $\log_{10}$ body weight in *Musca*

Table 2. Tests of significance between treatment groups in Table 1 for $\log_{10}$ esterase spot intensity calculated by Scheffe's S-test.

Treatment* means to be compared	Scheffe's S-test value	Significance** (1% level)	Inference
1 vs 2	5.2943	Significant	♀ TM different from ♂ TM
3 vs 4	13.2610	Significant	♀ VP different from ♂ VP
5 vs 6	4.5584	NS $p > 0.01$	No difference between ♂ & ♀ F_1 (TM × VP)
7 vs 8	4.2500	NS $p > 0.01$	No difference between ♂ & ♀ F_1 (VP × TM)
9 vs 10	9.4172	Significant	♀ UVP different from ♂ UVP
(5 + 6) vs (7 + 8)	0.2295	NS $p > 0.05$	Pooled ♂ + ♀ F_1 (TM × VP) not different from pooled F_1 (VP × TM)
3 vs 9	4.7689	NS $p > 0.01$	No difference between ♀ VP & ♀ UVP
4 vs 10	3.6479	NS $p > 0.05$	No difference between ♂ VP & ♂ UVP
1 vs (5 + 7)	19.6669	Significant	♀ TM different from pooled ♀ f_1's
2 vs (6 + 8)	18.0130	Significant	♂ TM different from pooled ♂ F_1's
(3 + 9) vs (5 + 7)	1.4880	NS $p > 0.05$	No difference between pooled ♀ VP and UVP and pooled F_1's
(4 + 10) vs (6 + 8)	3.5612	NS $p > 0.05$	No difference between pooled ♂ VP and pooled ♂ F_1
1 vs 3	21.1661	Significant	♀ TM different from ♀ VP
2 vs 4	13.1502	Significant	♂ TM different from ♂ VP

* See Table 1 for details of treatment groups.

** NS = Not significant at 1% level.
UVP = Unselected VP.

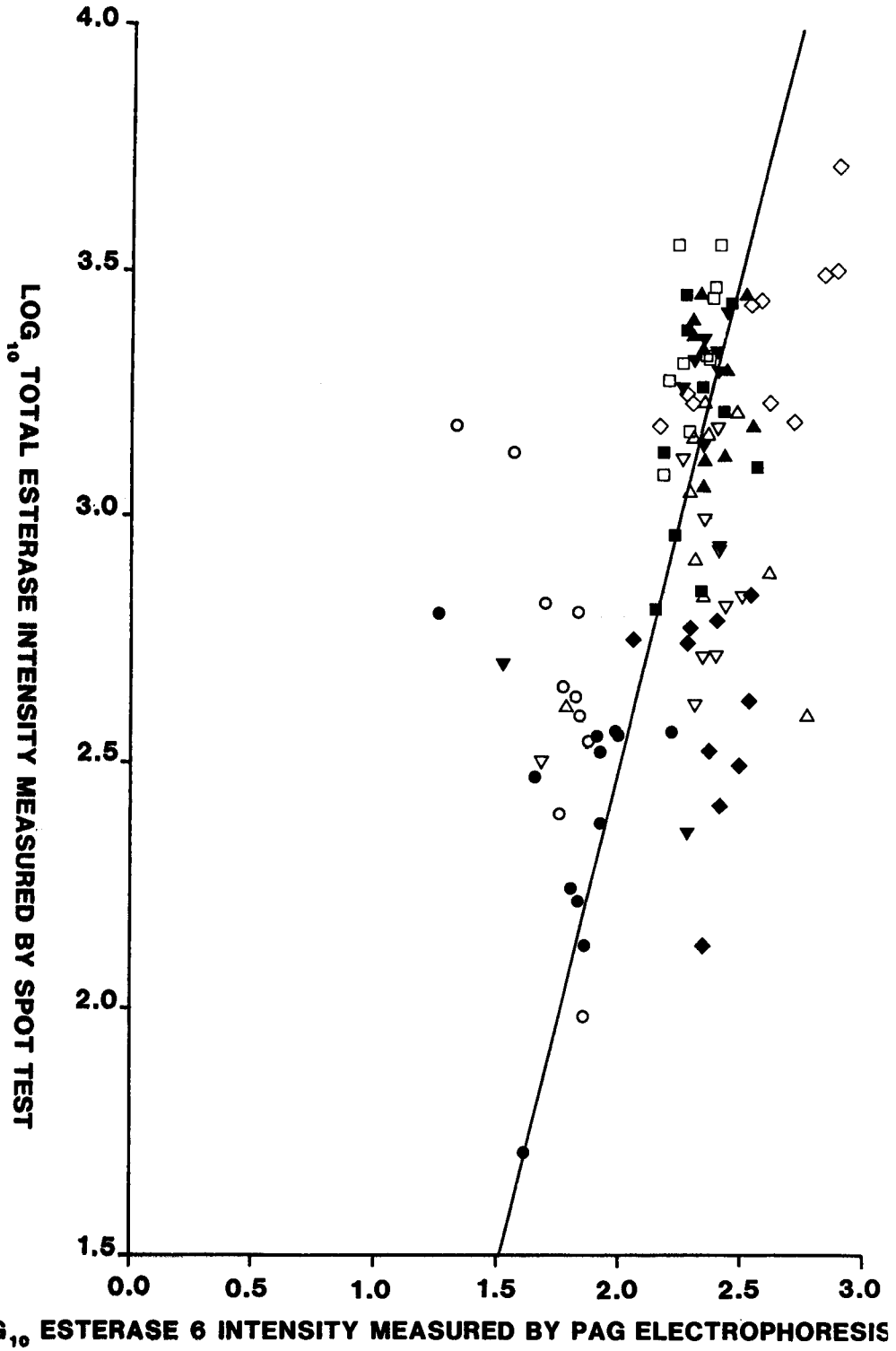

Fig. 1. Graph of the relationship between $\log_{10}$ Esterase 6 intensity measured by PAG electrophoresis and $\log_{10}$ total esterase intensity measured by the spot method. The regression line ($r = 0.5318$ $p<0.001$) is then calculated for all values shown. Different strains are represented as follows: ●○ = TM; ■□ = UVP; ◆◇ = VP; ▲Δ = F_1 (VP × TM); ▼∇ = F_1 (TM × VP). Males are shown as closed figures, females as open figures.

domestica, Sitophilus granarius (Lavadinho 1975, 1976) and *Tribolium castaenum* (Margham and Thomas 1980).

It would be most interesting to apply the test to samples from field populations of *Ae. aegypti* where spraying programs with organophosphate insecticides, particularly malathion, were in progress. From a practical point of view, the spot test needs few chemicals and simple inexpensive apparatus, which could easily be transported for sampling in relatively inaccessible areas.

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