MICROPLATE ASSAY ANALYSIS OF REDUCED FENITROTHION SUSCEPTIBILITY IN HAITIAN *ANOPHELES ALBIMANUS*

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ABSTRACT. Reduced fenitrothion susceptibility in Haitian *Anopheles albimanus* is documented using time/mortality measurements from otherwise standard World Health Organization (WHO) bioassays. Survival beyond a time threshold in bioassays is shown to be highly correlated with elevated non-specific esterase levels. A shift in resistance incidence from less than 20 to over 60% in a six-month period is documented using both the bioassay and microassay procedures, showing the potential of microplate assay methods in early detection of resistance. Conventional 24-hour reading of WHO bioassay data failed to detect resistance until the level reached 60%. Resistance appeared to be focal, with an increase in intensity coinciding with a fenitrothion spray cycle for malaria control.

INTRODUCTION

Until recently, simple field methods that detect resistance mechanisms in single insects have not been available. As a result, few detailed studies have been conducted on the spatial distribution of resistance levels produced by specific resistance mechanisms (Brown and Brogdon 1987). Presently, development of field resistance-detection methods based on the assay of enzyme activities in individual insects is proceeding along two lines. Biochemical microassays of resistance enzymes have been adapted for use on filter paper (Pasteur and Georghiou 1981) or in microtiter plate wells (Brogdon and Dickinson 1983, Hemingway et al. 1986, Brogdon and Barber 1987, Ashour et al. 1987, Brogdon 1988).

We incorporated microplate assay resistance detection methods into field studies in Haiti and Guatemala. In Haiti, where resistance to organophosphate insecticides was unknown, emphasis was placed on evaluating stability of reagents, developing procedures suited to primitive tropical conditions, and establishing susceptibility baselines for the microplate assays. In Guatemala, where widespread resistance based on agrichemical practices is present (Chapin and Wasserstrom 1981), field studies focused on the incidence and geographic distribution of organophosphate and carbamate resistance using microplate assay and World Health Organization (WHO) bioassay data from the same mosquito collections (Brogdon et al. 1988, Beach et al. 1988; C. Cordon-Rosales, R. F. Beach and W. G. Brogdon, unpublished data).

The advantage of resistance assays in single insects is the detection of resistance at low frequency in a population, allowing a better chance for corrective action (Brown and Brogdon 1987). For this role of microassay resistance detection to be documented, resistance must appear in an area where there are previously-established susceptibility baselines for the new microplate methods. In 1985, one of our study areas in Haiti provided that opportunity.

During 1984, entomologists from the Haitian Service National des Endemies Majeures (SNEM) noticed an apparent decline in effectiveness of the fenitrothion spray program (higher numbers of malaria cases) in the Les Cayes region of the southern peninsula of Haiti. We had last surveyed that area with microplate assay detection methods in 1981, finding no evidence of resistance. Since 1981, SNEM had regularly (bimonthly) conducted conventional WHO bioassays for fenitrothion susceptibility throughout the region, but had observed no evidence for resistance.

Early in 1985, frozen mosquito specimens from Les Cayes that had been returned to CDC (Atlanta) for other studies were microassayed for resistance. A major advantage of microplate resistance detection methods is that fresh or frozen insects may be used (Brogdon 1984a, Brown and Brogdon 1987). Significantly elevated (twice the maximum activity in susceptibles) non-specific esterase activity was detected in 10% of the individuals in a small sample (20 mosquitoes). These findings suggested that a fine opportunity existed to evaluate an emerging control problem using the new microplate resistance detection methods. As a result two short-term field studies were conducted six months apart that resulted in the findings reported herein. Confirmation (via electrophoresis, synergism and metabolism studies) of elevated esterases as a fenitrothion resistance mechanism in *Anopheles albimanus* in Haiti and Guatemala will be published elsewhere (W. G. Brogdon and A. M. Barber, unpublished data).

MATERIAL AND METHODS

Study areas. *Initial studies.* Study sites for field-adapting microplate resistance assays and

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establishing susceptibility baselines are shown in Fig. 1. In the Les Cayes region (area 5) only the Mairier site was evaluated prior to 1985. Sites were chosen for accessibility and correspondence with major areas of rice culture throughout the country. *Anopheles albimanus* Wiedemann is a tropical lowland mosquito that thrives where rice is grown asynchronously, as in Haiti, and is the principal malaria vector in the Central American and Caribbean regions (Breeland 1972). Sites were studied sequentially through the period 1981 to 1984.

1985 studies. Following detection of elevated esterase activity, villages were chosen that broadly encompassed the Les Cayes region of the south peninsula (Fig. 1, Les Cayes, area 5). Sites lay on an east-west line of 70 km, roughly centered on Les Cayes (the city). One site 50 km to the north (Maniche) was sampled in October to determine the northward extent of the suspected resistance area. Beyond Maniche, mountainous terrain provides a natural boundary to *Anopheles albimanus* breeding. Most sites were sampled in both May and October.

Mosquito collections. Man-biting collections of *An. albimanus* were made by SNEM personnel in rice fields adjoining study villages during the dusk biting activity peak. On the average, 40 to 50 mosquitoes were captured by each collector in a one-hour period. Collection rates around Les Cayes were higher than at other sites in the country, probably due to the more intensive rice cultivation in that region. Collected mosquitoes were provided sugar solution (2%) and held overnight.

Resistance assay methods. *Bioassay.* The WHO adult mosquito resistance test kit was used in all bioassays. Although insecticide exposures were conducted using current 1% fenitrothion-impregnated papers as recommended by WHO, SNEM personnel routinely limit exposure times to one hour rather than the recommended two; the fenitrothion discriminating dose recommended by WHO for anophelines (species unspecified) is too high for *An. albimanus.* The WHO acknowledges that discriminating dosages must be adjusted based on the species (or even population) of mosquito (World

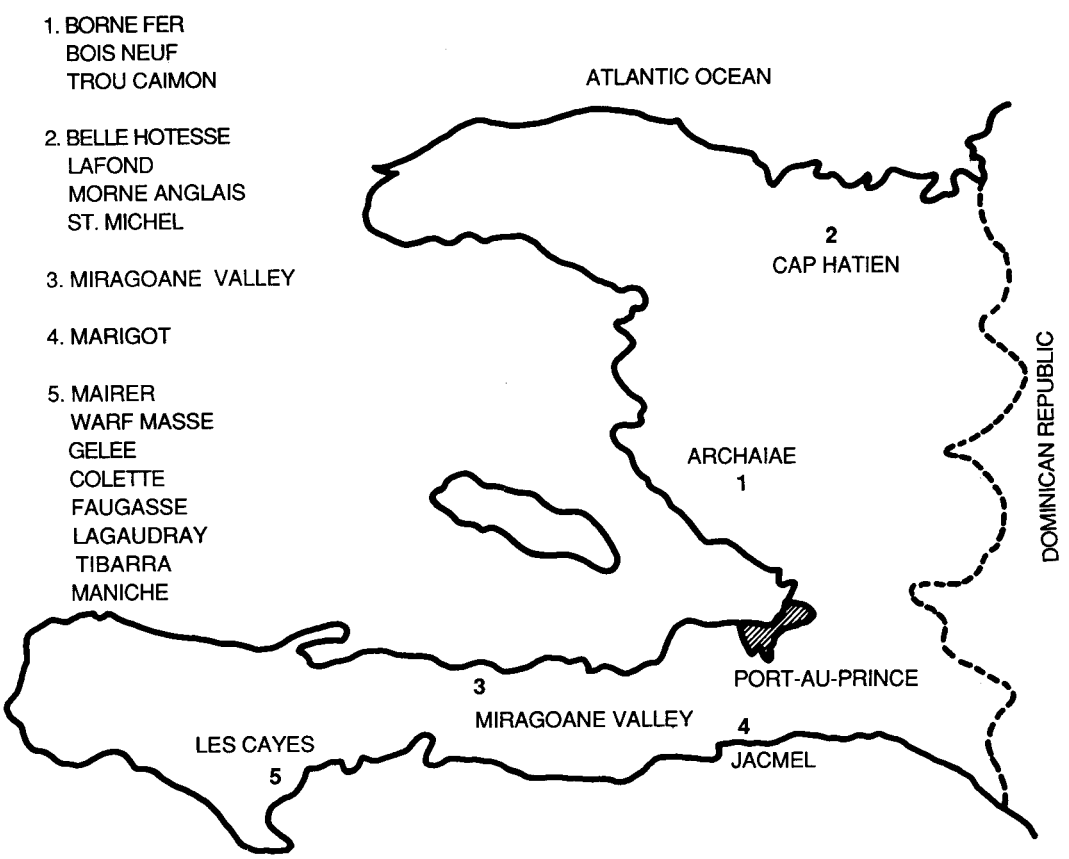

Fig. 1. Map of Haiti showing study areas. Numbers refer to areas labelled on map. Study villages are listed by area number.

Health Organization 1986). Since 1981, we have used a further modification of the WHO test for research purposes. We have observed repeatedly in Haiti and in Guatemala that fenitrothion knockdown is equivalent to mortality, facilitating the use of time-mortality data to evaluate susceptibility. As SNEM personnel conducted the bioassays, knockdown (=mortality) rates were recorded at 30-minute intervals for two hours following the end of exposure. That is, we simply collected more data than is customary in the WHO test. For each study site, one control tube and four tubes containing fenitrothion-impregnated papers were run, each tube containing 25 adult female mosquitoes. Mortality was also recorded 24 hours postexposure to fenitrothion.

Enzyme microassay. For each study site, enzyme microassays and WHO bioassays were run simultaneously on mosquitoes from the same pools. Mosquitoes were individually homogenized in 100 µl of 0.05 M, pH 6.8 potassium phosphate buffer and diluted to 1 ml. Homogenate aliquots of 100 µl were transferred to microtiter plate wells. At least 50 mosquitoes were microassayed for each site. Once the microtiter plates were loaded with homogenate, four replicates of two types of resistance enzyme microassays (non-specific esterase and insensitive acetylcholinesterase) were run on each mosquito.

Assays were run as described by Brogdon and Dickinson (1983). In assays for elevated non-specific esterase activity, 100 µl of B-naphthyl acetate (56 mg/10 ml 2-propanol/90 ml buffer) were added to each well as the esterase substrate using a 96-trip transfer plate (Vaccupette, Research Products International Corp., Mount Prospect, IL). Following a 10-minute incubation (Beach et al. 1988) at ambient temperature, (25–27°C in May and October) 100 µl of dianisidine dye reagent (100 mg/100 ml water) were added to each assay well. Dianisidine forms a chromophore (absorbance maximum of 550 nm) with the B-naphthol produced by esterase hydrolysis of B-naphthyl acetate. Mosquitoes with elevated non-specific esterases produced assays with a purple color as opposed to pink in assays of mosquitoes with normal esterase levels. Although results could be scored by eye, a portable enzyme immunoassay (EIA) reader (Minireader II, Dynatech Laboratories, Alexandria, VA) was used for precise quantitation. A provisional susceptibility threshold of absorbance 550 = 0.9 was used. This value is twice that of the mean and is slightly above the range of values we have determined for susceptible *An. albimanus* populations.

The Ellman (1961) method, as modified by Brogdon and Dickinson (1983), was used for microassays of AChE. Insensitive enzyme was detected using molar propoxur in the substrate solution. A 100-µl portion of acetylthiocholine iodide (ACTH, 75 mg/10 ml acetone/90 ml buffer) substrate solution was added to each assay well, followed by 100 µl 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB, 13 mg/100 ml buffer). In test wells, propoxur (21 mg/100 ml substrate solution) was included in the acetone portion of the substrate solution. No temperature corrections were required in these tests since, as has been observed in field microplate assays of human blood cholinesterase (Brogdon 1987) temperature effects on AChE assays run in microplates are minimal within the temperature ranges generally encountered. However, temperatures above 37°C might require corrections in assays (unpublished data). After 30 minutes, the intensity of the yellow chromophore produced by DTNB reaction with the thiocholine hydrolysis product was scored by eye and read at 410 nm using the EIA reader. In this assay, detection is based on failure of propoxur to inhibit insensitive forms of ACHE, resulting in yellow wells rather than colorless ones.

Protein levels for each mosquito were determined by a microplate assay method (Brogdon, 1984b, 1984c). Plates were loaded with homogenate as described above. Portions (200-µl) of diluted dye reagent (Bio-Rad Laboratories, Richmond, CA) were added to each well using the transfer plate. Results were scored by eye or read at 595 nm using the EIA reader.

Combined experiments. In bioassay tests run for this purpose, mosquitoes at 135 minutes postexposure were subjected to non-specific esterase microassay. Data for surviving mosquitoes and those killed by the insecticide were compared. In addition, mortality rates and esterase levels were closely compared for each study site to determine if data from the two types of resistance assays could be correlated.

RESULTS AND DISCUSSION

Bioassays. Before October 1985, there was no evidence for reduced fenitrothion susceptibility in the routine bimonthly screening by SNEM using 24-hour mortalities. Several thousand mosquitoes from the Les Cayes region were tested each year. In October, a small number of survivors (<5%) was noted after 24 hours. In tests using mortality versus time as the criterion, a progressive decline in mortality was seen in May and October 1985, compared to the mortality versus time baseline established at Mairier in 1981 (Fig. 2).

The data in Fig. 2, which show a simple mortality versus time relationship, may also be plotted on probit paper as the log percent mortality versus time (data not shown). The LT_{50}, or time

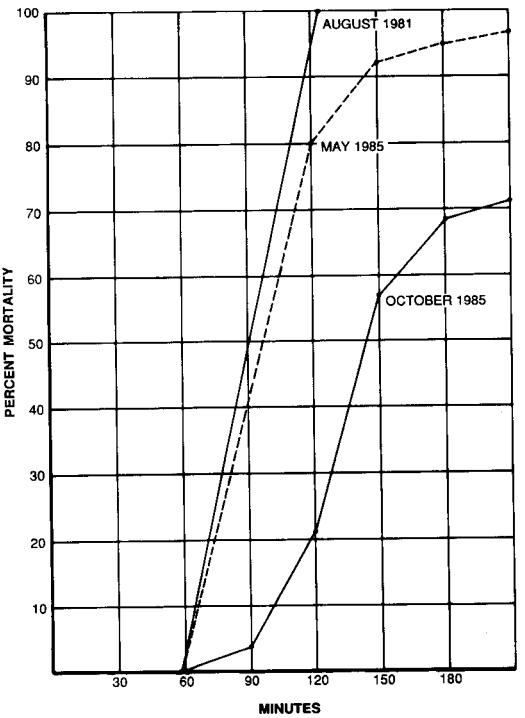

Fig. 2. Combined bioassay time/mortality data (1,750 mosquitoes) for eight study sites in the Les Cayes area. Data for 1981 are from Mairier and represent a susceptible baseline.

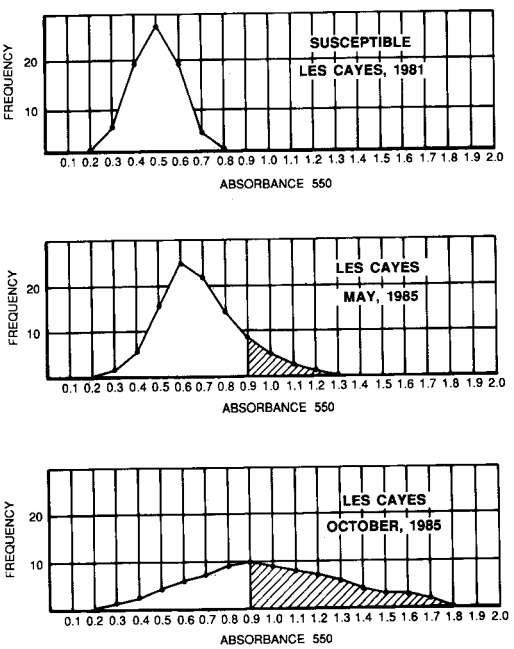

Fig. 3. Combined non-specific esterase microassay data for the eight study sites. Data for 1981 (susceptible baseline) are from Mairier. Results for Les Cayes represent data (four replicates/mosquito) for 742 mosquitoes. Frequencies are percentages. In figures 3–5, values in the cross-hatched areas lie above our threshold for resistance.

when 50% of the mosquitoes have died, values derived from these plots are no different from those inferred from Fig. 2. That is, in 1981, May 1985, and October 1985, the LT_{50} values are 90, 98 and 145 minutes, respectively. For field use, we prefer the plot shown in Fig. 2, since probit paper is not readily accessible in field locations such as Les Cayes, or even Port-au-Prince.

The time limit for survival of susceptible *An. albimanus* on 1% papers in Haiti (as well as in Guatemala) was 130 minutes after exposure was initiated (for example, Fig. 2, Mairier data). The percentage of mosquitoes surviving at or beyond 135 minutes was recorded as the percentage of less susceptible individuals. The average percentages of less susceptible mosquitoes near Les Cayes in May and October 1985 were 19 and 49% respectively, as compared with 0% in 1981 (Fig. 2).

Microassays. Prior to 1985, all microplate assays of non-specific esterase showed normal levels of activity (Fig. 3, Les Cayes; Mairier 1981) and there was no evidence of insensitive acetylcholinesterase (data not shown). In May and October, 1985, there was a progressive increase in frequency of mosquitoes which showed elevated levels of non-specific esterase activity (Fig. 3). In addition, in both May and October, a small percentage (<0.2% of individuals were positive for the insensitive acetylcholinesterase mechanism (data not shown). Protein levels were only weakly correlated (correlation coefficient = 0.6) with esterase levels in 1981 and 1985. For interpretation of microassay results, a susceptibility threshold of $absorbance_{550}$ = 0.9 was used in these experiments. This value is twice the mean value for susceptibles (Fig. 3, Les Cayes (Mairier) 1981) and all values for susceptibles fell below 0.9. The percentage of mosquitoes with absorbances above 0.9 was recorded as the percentage of individuals with elevated esterase. The average percentages of mosquitoes with elevated esterase near Les Cayes in May and October 1985 were 19 and 45% respectively as compared with 0% in 1981 (Fig. 3).

Correlation of bioassay and microassay results. Comparative data for estimated bioassay resistance percentages and elevated esterase incidence percentages are shown in Table 1 for the eight study sites near Les Cayes. Survival beyond 135 minutes was highly correlated with elevated non-specific esterase activity (correlation coefficient by least squares = 0.9584, r^2 = 0.9186). Sites with highest resistance showed levels of around 60%. For all sites, there was

close agreement between bioassay and microassay results.

In a separate experiment using mosquitoes from Lagaudray in October, mosquitoes killed and those surviving at 135 minutes after fenitrothion exposure was initiated were subjected to elevated esterase microassay (Table 2). Although esterase levels were reduced by fenitrothion inhibition, the close association between survival and higher esterase level is apparent.

Expansion of a resistance focus? In Guatemala, resistance may be focal; sites 40km apart may show widely differing levels of resistance or predominance of different resistance mechanisms (Brogdon et al. 1988). The data from Les Cayes may reveal a May resistance focus which had increased in area by October. In May, all the sites surrounding Les Cayes showed similar resistance profiles (resistance level = 12.6 ± 5.3%) except one, Gelee (resistance level = 43%). The respective LT_{50} levels figured from probit plots are 95 ± 2 minutes (all sites) and 145 minutes (Gelee). Frequencies of elevated esterases are shown for a representative low resistance site (Mairier, Fig. 4) and Gelee (Fig. 5) showing the much higher frequency at Gelee. Consider also that those sites which showed the greatest increase in resistance level between May and October (Mairier, Warf Masse, Lagaudray, Faugasse) were the closest to Gelee. Collette (west), Tibarra (east), and Maniche (north) were at the limits of the study area, while Gelee is near the city of Les Cayes. The increase in resistance level between May and October coincided with a fenitrothion spray cycle conducted by SNEM throughout the Les Cayes region between August and October. That this spraying contributed to the increased resistance level seems likely though unproven, given the absence of data on simultaneous insecticide use for rice culture in the area. There is evidence that feni-

Table 1. Estimated percentages of elevated esterase and prolonged survival in bioassays in *Anopheles albimanus* using microassay and bioassay methods on the same mosquito collections from eight study sites near Les Cayes.

	Biochemical microassay		Modified WHO tube tests	
Location	May	Oct.	May	Oct.
Mairier	11%	64%	10%	62%
Galee	40	59	46	58
Warf Masse	15	55	12	63
Lagaudray	16	65	14	50
Tibarra	14	8	—	15
Faugasse	17	64	14	—
Colette	—	27	—	21
Maniche	—	15	—	14
Total sample	19	49	19	45
	n = 742 mosquitoes		n = 1,750 mosquitoes	

Coefficient of correlation between microassay and tube test data = 0.9584, $r^2 = 0.9186$.

Table 2. Comparison of esterase levels for mosquitoes (n = 125) surviving or not surviving 135 minutes in the modified bioassays. Results are percentages.

	Bioassay	
Microassay $absorbance_{550}$	Killed by 135 min.	Survivors at 135 min.
Below 0.5	51.2	1.6
Above 0.5	0	47.2

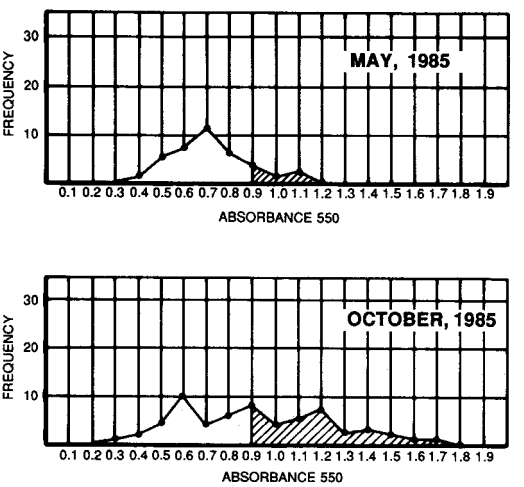

Fig. 4. Microassay data from May and October 1985 for the Mairier study site. Frequencies are percentages.

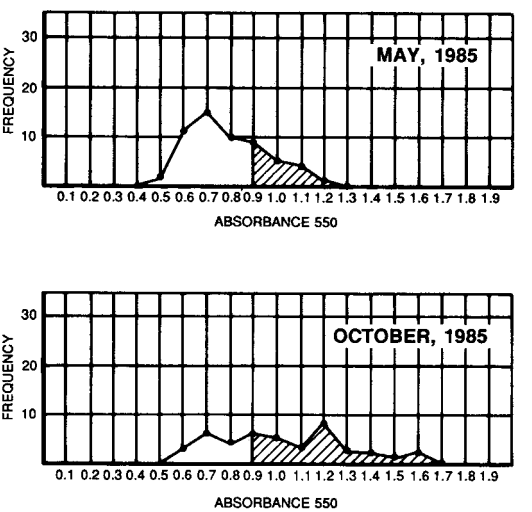

Fig. 5. Microassay data from May and October 1985 for the Gelee study site. Frequencies are percentages.

trothion exposure among the spraymen was exceptionally high (Brogdon 1987) during this spray cycle and overtreatment with insecticide may have occurred. Further study of this intriguing question was prevented by the outbreak of civil unrest in Haiti.

The non-specific esterase (α or β naphthyl acetate substrate) resistance mechanism is widespread in insects and has been thoroughly studied in *Culex* (Curtis and Pasteur 1981, Fournier et al. 1985, Villani et al. 1983). Prior to these studies and those in Guatemala this mechanism had not been implicated in anopheline resistance to insecticides. Malathion carboxylesterases that show no reactivity toward the naphthyl acetates have been shown to cause malathion resistance in *An. arabiensis* Patton and *An. subpictus* Grassi (Hemingway 1982, 1983). Hemingway et al. (1986) also observed elevated nonspecific esterase levels in some Sri Lankan *An. nigerrimus* Giles, but found no correlation with resistance.

Naphthyl acetates (α and β) are not the definitive substrates for carboxylester hydrolase activity; in fact, they are general substrates for a variety of other hydrolases, including arylester, hydrolase, acetylester hydrolase and cholinesterases (Heymann 1980). It would be wise, therefore, to make no assumptions about the potential for cross-resistance of the esterase described in these studies, pending further investigation.

In the course of these experiments, experience was gained in using the non-specific esterase and insensitive acetylcholinesterase microplate assays under tropical conditions in the field. Reagents preweighed and kept over silica gel remained stable at ambient temperatures for at least two weeks. It was important to keep dianisidine in foil-wrapped vials and to use this reagent immediately after the working solution was prepared. Individuals trained as field entomologists for the malaria control program ran all assays, once the procedures were demonstrated. For the microplate assay approach to resistance detection to be effective in countries with limited resources, the tests must be conducted by individuals with the same level of training as those who have routinely run bioassays.

The method proved to be easily taught, fast, accurate, and less time-consuming than the bioassay. The advantages to seeing specific resistance mechanisms in individual mosquitoes in the field should make field studies of resistance more practical and informative. Many researchers are now working on biochemical/immunological methods of resistance detection; these methods represent a promising new approach for analysis of resistance problems (Brown and Brodgon 1987).

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