Research

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Microplate assay analysis of the distribution of organophosphate and carbamate resistance in Guatemalan *Anopheles albimanus*

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Simple microplate assay methods for determining the frequency of insecticide resistance in single mosquitos were used to study the distribution and localization of organophosphate and carbamate resistance in field populations of Anopheles albimanus *Weidemann in Guatemala, where such resistance, caused by heavy use of agricultural pesticides, has long been assumed to be widespread. Areas of complete susceptibility to organophosphates and carbamates were observed, as well as areas where the resistant phenotypes represented up to 98% of the population. Overall, the resistance levels were lower and more localized than expected. Two mechanisms of resistance were identified by the microassay methods. These were the elevated esterase (nonspecific esterase) and insensitive acetylcholinesterase mechanisms which were selected independently, the former (documented for the first time in Central American anophelines) being predominant. These methods represent a promising new technology for the detection and assessment of resistance and will facilitate improved control strategy decisions.*

The malaria vector, *Anopheles albimanus*, from the coastal areas of Central America has shown resistance to nearly all known insecticides through the development of a variety of resistance mechanisms. Davidson was the first to review organochlorine multiresistance in this vector; resistance to both DDT and dieldrin in the same mosquito populations appeared in Central America in the late 1950s (*1*). Subsequently, resistance to organophosphates and carbamates appeared, for which one mechanism has been described (*2–7*). Synthetic pyrethroid resistance has also recently been reported in Guatemala (*8*). This rise and spread of resistance in Central America has been attributed to the rapidly increasing use of agricultural pesticides (*9*).

Most studies of resistance in *A. albimanus*, particularly concerning the biochemical mechanisms of resistance, have been evaluated in laboratories on colonized, selected mosquito strains because of the lack of a methodology for ascertaining resistance mechanisms in the field. As a result, the practical (operational) field impact of these studies on Central American *A. albimanus* has been minimal. For

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example, there have been no studies of the spatial distribution of resistance mechanisms or levels, and the mechanisms that were identified in the laboratory have not been confirmed in the field.

For decades, field studies on resistance problems *in situ* have relied on the WHO bioassay kit for determining resistance in mosquitos. Although much has been learned from this detection system, it has several inherent limitations (*9*). Only one insecticide can be tested per insect and, without a known discriminating dosage, large numbers of insects are needed to generate probit lines. Using discriminating dosages allows detection of resistance with smaller numbers of insects but not determination of the resistance level or mechanism. Moreover, discriminating dosages that could be applied to all mosquitos do not exist. Also, false positives may occur because of deteriorating filter-papers or precedural variables, such as temperature or humidity. Finally, the bioassay methods are ineffective in detecting resistance phenotypes at low frequencies.

For these reasons, we proposed the development of microplate assay methods for detecting resistance in single mosquitos[a] based upon methods we had developed (*10*) and were using in resistance studies.[b] Since then, workers in several laboratories have developed or are developing similar biochemical and new immunological methods for evaluating resistance (*11–15*). Recently, these methods have been introduced into field studies of resistance in the anopheline vectors of malaria in Haiti and Sri Lanka (*16*).

This paper describes the results of a microplate assay method that was used to detect and assess the spatial distribution of organophosphate and carbamate insecticide resistance in Guatemalan *A. albimanus* in various parts of the country. Other studies on the relationship between the microplate assay and the WHO bioassay, on the temperature effects in microplate assays, and on longitudinal studies of resistance foci are under way.

MATERIALS AND METHODS

Mosquito collections

The spatial distribution of resistance was determined on specimens of *A. albimanus* that were collected throughout the country from selected locations. The mosquitos were collected in three ways. (1) Human-biting collections were made in the early evening in sites that were distant from corrals since *A. albimanus*, a zoophilic species, rarely bites man in close proximity to cattle. (2) In the corrals, mosquitos that had virtually all been blood-fed were collected from their resting sites in vegetation or other structures (*17*). (3) Mosquitos were also collected using ultraviolet light updraft traps (*18*). The collections decreased in size in the order: corral captures > human-biting captures > UV-trap captures.

The live mosquitos were immobilized using a dry-ice chest and transferred to labelled vials that were returned to the laboratory on dry ice for storage at −70 °C. Collections of more than 200 *A. albimanus* females were obtained from each study site.

Microassays and experimental design

Elevated esterase microplate assay. Individual mosquitos were homogenized in 100 μl of 0.05 mol/l potassium phosphate buffer, pH 6.8, and diluted to 1 ml with buffer. Aliquots of 100 μl were used for each assay replicate. The microplate assay procedure of Brogdon & Dickinson was used for enzyme assays (*10*). To each 100 μl of homogenate were added 100 μl of β-naphthyl acetate (56 mg/10 ml 2-propanol/90 ml buffer) using a 96-tip transfer plate[c] and the preparation was incubated at ambient temperature for 10 minutes (in Guatemala City, the laboratory temperature was 25 °C). A 100 μl aliquot of dianisidine (100 mg/100 μl water) was then added. Absorbances were read at 550 nm in an enzyme immunoassay reader[d] or evaluated visually.

Insensitive acetylcholinesterase microplate assay. Individual mosquitos were homogenized in 100 μl of 0.05 mol/l potassium phosphate buffer, pH 6.8, and diluted to 1 ml with buffer. Although the pH optimum for the original acetylcholinesterase assay of Brogdon & Dickinson was 7.4, the use of a buffer with a pH of 6.8 did not affect the detection efficiency and allowed both the elevated esterase and the insensitive acetylcholinesterase microassays to be more easily run with replication on the same mosquitos (*10*). To each assay well were added 100 μl acetylthiocholine iodide (75 mg/100 ml buffer) containing 0.1 mmol/l propoxur and 100 μl Ellman's reagent (DTNB, 13 mg/100 ml buffer). Absorbance (at 414 nm) was measured at 30 min using the microplate reader. Reaction kinetics may also be monitored using the same system (19).

Protein microplate assay. The significance of size variations between mosquitos for the interpretation

[a] BROGDON, W. G. *A proposed new method under development for field detection and evaluation of insecticide resistance.* Unpublished WHO document VBC/84.859.

[b] BROGDON, W. G. *New methods for biochemical field studies of insecticide resistance.* Paper presented at the annual meeting of the American Mosquito Control Association, Sacramento, CA, 1982.

[c] Vaccupette, Research Products International Corp., Mount Prospect, IL, USA. Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

[d] Minireader II, Dynatech Laboratories, Alexandria, VA, USA.

of results was evaluated using a microplate protein assay (*20, 21*). To 100 μl aliquots of mosquito homogenate were added 200 μl of diluted dye reagent.[e] Absorbance (at 600 nm) was noted using the microplate reader, and values were compared with a standard curve.

For each mosquito, three replicates were assayed (for the elevated esterase, insensitive acetylcholinesterase, and protein). The microtitration plates were organized so that thirty-two mosquitos (three replicates) could be microassayed using one of the three types of assays on a single plate. Thus, three plates were used for running the three assays on the same thirty-two mosquitos, and the transfer plates could be used to maximum advantage.

Resistance frequencies. Estimates of the percentage resistance were made from comparative bioassay/microassay data collected from Guatemalan *A. albimanus*. The resistance thresholds in microplate assays of Guatemalan *A. albimanus* are (at absorbance 550) $\geqslant 0.9$ in the elevated esterase assays and (at absorbance 410) >0.3 in the insensitive acetylcholinesterase assays.

[e] Bio-Rad Laboratories, Richmond, CA, USA.

RESULTS

Approximately 1100 mosquitos were microassayed for the elevated esterase and insensitive acetylcholinesterase mechanisms. The protein levels were sufficiently consistent and no corrections for mosquito size variation were needed. Data were organized according to the Guatemalan administrative departments in which the study sites were located (Table 1, Fig. 1).

Neither of the two resistance mechanisms was detected in non-agricultural areas and where there had been little or no use of organophosphate or carbamate insecticides for malaria control. Both mechanisms were detectable but uncommon in areas where agriculture was relatively light (Fig. 2, 3); higher frequencies of both resistance mechanisms were observed throughout the heavily agricultural areas near the Pacific coast (Fig. 4, 5).

Frequency distributions for elevated esterase absorbance values from the Pacific coastal areas revealed three absorbance peaks (Fig. 4) which correspond to susceptible or resistant homozygotes and

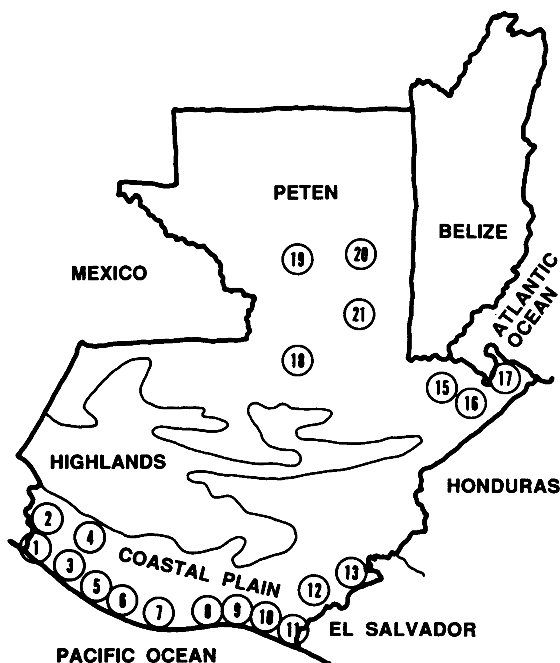


Fig. 1. Map showing the 21 collection sites in Guatemala; at least 200 mosquitos were collected from each location (total collection, 9417 mosquitos). Table 1 gives the name of the department for each site number.

Table 1. Locations of the 21 study sites by administrative department and intensity of agriculture in the area

Site number/ department	Location	Intensity of agriculture
1, 2/San Marcos	Pacific coast/Mexican border: Ocos, Hacienda la Zarca	Heavy
3, 4/Retalhuleu	Pacific coast: Colonia la Felicidad, Hacienda la Agricola	Heavy
5, 6, 7, 8/Escuintla	Pacific coast: Aldea San Pedro Nahualate, Aldea Santa Marta el Mar, Aldea Empalizada, Barrio el Mangelar	Heavy
9, 10/Santa Rosa	Pacific coast: Aldea el Chapeton, Aldea la Avellana	Heavy
11, 12, 13/Jutiapa	Salvadoran border: Parcelamiento Montufar, Aldea el Toro, Aldea las Moritas	Light
14/Guatemala	Foothills near Guatemala City: Finca Rancho Grande	Light
15, 16, 17/Izabal	Atlantic coast: Hacienda las Vegas, Aldea el Relleno, Aldea San Francisco II	Light
18/Alta Verapaz	Northern rain forest: Aldea Chajmaic	None
19, 20, 21/Petén	Northern rain forest: Finca Esquipulas, Aldea el Quetzal, Aldea Los Angeles	None

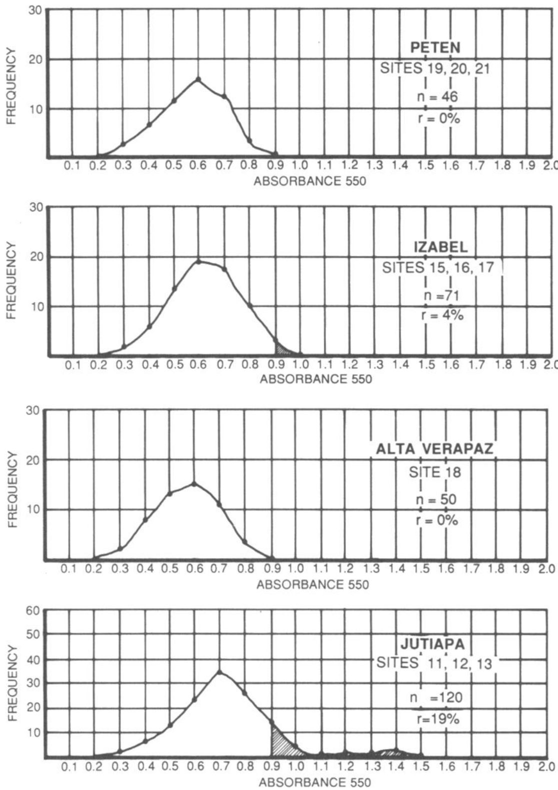


Fig. 2. Elevated esterase microassay data from four Guatemalan departments with little or no agricultural spraying. Frequencies (at absorbance 550) were based on four replicates per mosquito (n = sample size; r = microassay resistance level determined by the described criteria).

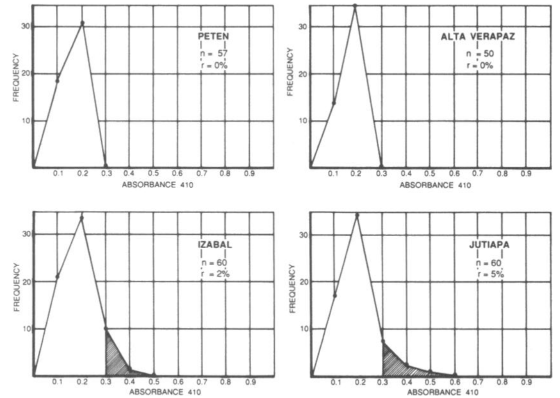


Fig. 3. Insensitive acetylcholinesterase microassay data from four Guatemalan departments with little or no agricultural spraying. Frequencies (at absorbance 410) were based on three replicates and one control replicate per mosquito (n = sample size; r = microassay resistance level determined by the described criteria).

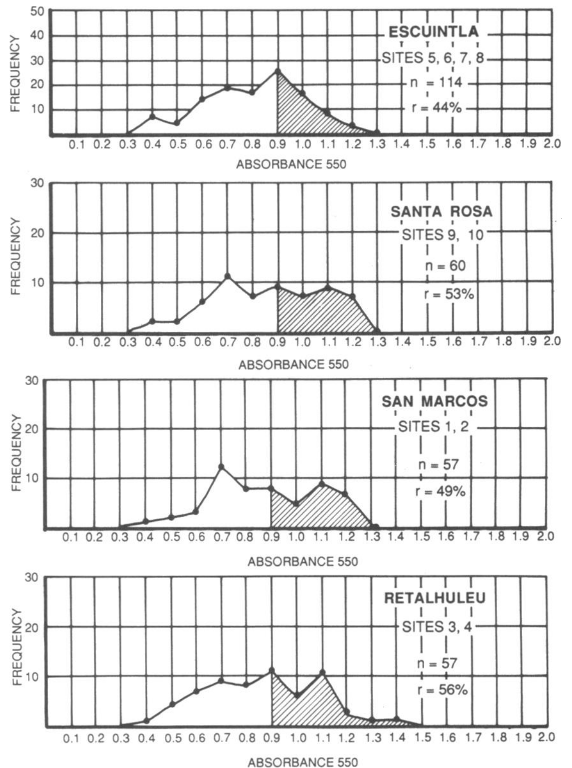


Fig. 4. Elevated esterase microassay from four Guatemalan departments with heavy agricultural spraying. Frequencies (at absorbance 550) were based on four replicates per mosquito (n = sample size; r = microassay resistance level determined by the described criteria).

heterozygotes, although certain sites did not show all three. The elevated esterase data from the Pacific coast were pooled, and the resulting frequency distribution was plotted (Fig. 6); the peaks (at absorbance 550) at 0.7, 0.9, and 1.1 correspond to the pink, lavender, and purple hues observed in the assays. The frequency distribution for insensitive acetylcholinesterase absorbance values from mosquitos in Escuintla (Fig. 5) shows a second peak in frequency at 0.4 (heterozygotes); the susceptible homozygote peak is at 0.2.

The distribution of these resistance mechanisms has been found to be geographically localized. For example, as shown in Fig. 7, a site near Lake Amatitlan showed a higher frequency of elevated esterase resistance than the area with the most resistance on

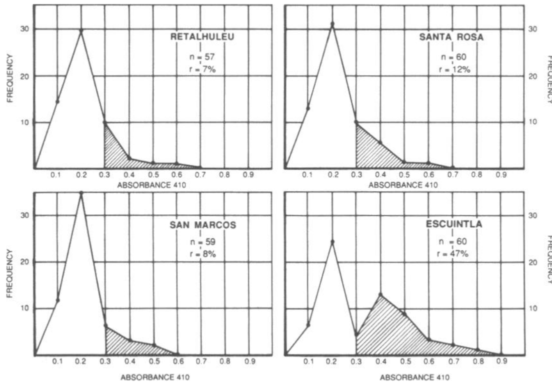


Fig. 5. Insensitive acetylcholinesterase microassay data from four Guatemalan departments with heavy agricultural spraying. Frequencies (at absorbance 410) were based on three replicates and one control replicate per mosquito (n = sample size; r = microassay resistance level determined by the described criteria).

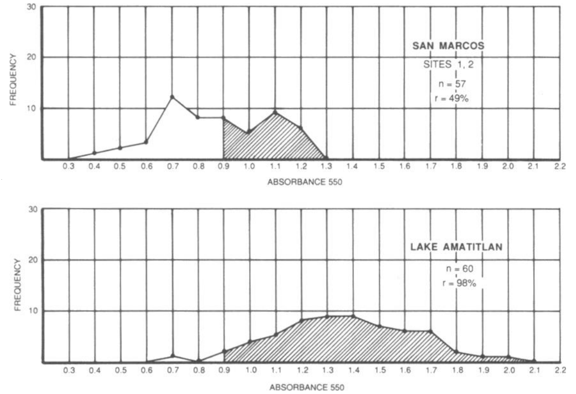


Fig. 7. Comparison of the frequencies and levels of elevated esterase microassay absorbances from two Guatemalan *A. albimanus* collection sites. Frequencies (at absorbance 550) were based on four replicates per mosquito (n = sample size; r = microassay resistance level determined by the described criteria).

the Pacific coast, San Marcos, which might be accounted for by intense, indiscriminate insecticide spraying of surface waters by the owners of lakeside vacation villas. Foci for insensitive acetylcholinesterase resistance were identified in Escuintla. Two sites, Puerto San Jose and Santa Marta el Mar, showed much higher frequencies of insensitive acetylcholinesterase than the other sites in Escuintla (Fig. 8) or elsewhere in Guatemala. Investigations revealed very large (33 km^2) cotton fields adjoining the breeding sites where insecticide was applied by frequent aerial spraying during the season.

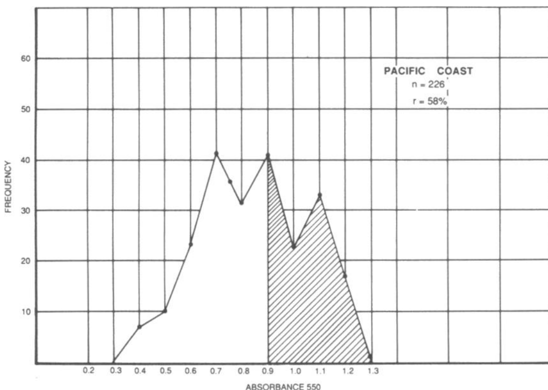
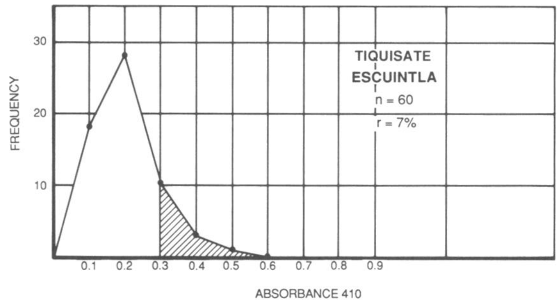


Fig. 6. Pooled elevated esterase microassay data for the Pacific coastal areas. Frequencies (at absorbance 550) were based on four replicates per mosquito (n = sample size; r = microassay resistance level determined by the described criteria).



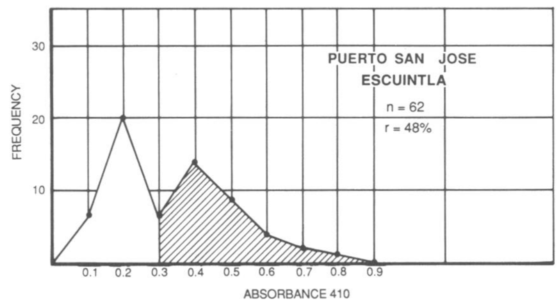


Fig. 8. Comparison of frequencies and levels of insensitive acetylcholinesterase microassay absorbances from two Guatemalan *A. albimanus* study sites in Escuintla. Frequencies (at absorbance 410) were based on three test replicates and one control replicate per mosquito (n = sample size; r = microassay resistance level determined by the described criteria).

Certain sites showed interesting differences in frequencies of the two resistance mechanisms. For example, in Escuintla, resistant homozygotes were missing from the elevated esterase microassay frequency distribution, but the insensitive acetylcholinesterase mechanism was present at relatively high frequency. In San Marcos, the insensitive acetylcholinesterase mechanism was at relatively low frequency but resistant homozygotes occurred at relatively high frequency.

DISCUSSION

Until the present study, the only organophosphate/carbamate resistance mechanism known in Central American *A. albimanus* was related to insensitive acetylcholinesterase (*22*). An elevated esterase mechanism similar to that detected in Guatemala has recently been reported in Haitian *A. albimanus*, but in no other anophelines. However, we have observed this mechanism in the field in *A. crucians* in Haiti and *A. pseudopunctipennis* in Guatemala (Brogdon & Beach, unpublished data). Others have found that antibodies raised to *Culex* resistance esterases did not react with esterases in a number of anophelines, but the epitope(s) involved in that study are unknown and may not be broadly diagnostic for resistance esterases (*23*).

It appears that both the heterozygotes and the homozygous susceptible and resistant mosquitos were detected using the elevated esterase assay. However, great caution must be exercised in analysis of isolated data showing only one genotype, since multiple copies of resistance genes may complicate analysis of otherwise simple dominant gene resistance (*24, 25*). Few homozygous resistant genotypes were detected in the insensitive acetylcholinesterase assays, which is to be expected with such low levels of resistance.

Both resistance mechanisms studied in Guatemala appear to be closely associated with heavily agricultural areas, but these are also the areas of highest mosquito densities and heaviest use of insecticides by the public health departments. Further studies will be necessary to delineate the relative roles played by agricultural and public health pesticide application in modulating the resistance patterns observed at particular locations at particular times. Selection for resistance for public health spraying has been implicated in field studies of *A. albimanus* in Haiti and *A. culicifacies* in Sri Lanka (*16*).

The most important issue of operational significance raised by the Guatemalan data is that resistance distribution is heterogeneous. First, the two mechanisms (elevated esterase and insensitive acetylcholinesterase), aside from their association with agricultural areas, appear to be selected independently. Areas where one mechanism is common do not necessarily have high levels of the other, as shown by data from San Marcos and Puerto San Jose. Also, resistance due to either mechanism may be highly localized and examples are Tiquisate vs. Puerto San Jose, Lake Amatitlan vs. San Marcos. Finally, the patterns of local insecticide use are associated with resistance foci, as illustrated by the data from Lake Amatitlan and Puerto San Jose.

Recognition of these differences in resistance distribution was facilitated through use of our microplate assay methods (*10*), which through further studies could help resolve resistance problems in the field.[f] The ability to spot low frequency resistance genotypes for specific mechanisms will make possible the earlier detection of resistance and precise studies of resistance microepidemiology. Most importantly, these methods will ultimately make resistance management techniques available to malaria control personnel in the field.

[f] See footnote *a*, page 340.

ACKNOWLEDGEMENTS

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RÉSUMÉ

ANALYSE PAR TITRAGE SUR MICROPLAQUE DE LA DISTRIBUTION DE LA RÉSISTANCE AUX ORGANOPHOSPHORÉS ET AUX CARBAMATES CHEZ *ANOPHELES ALBIMANUS* AU GUATEMALA

Afin d'étudier la distribution et la localisation de la résistance aux organophosphorés et aux carbamates chez des populations sauvages d'*Anopheles albimanus* Weidemann au Guatemala, où l'on soupçonne cette résistance, due

à un usage intensif de pesticides agricoles, d'être largement répandue, on a utilisé des méthodes simples de titrage sur microplaque permettant de déterminer la fréquence de la résistance aux insecticides chez des moustiques isolés. D'autres études sur la relation entre les résultats du titrage sur microplaque et ceux du titrage biologique OMS sont également en cours, ainsi que des études longitudinales sur les foyers de résistance. On a observé aussi bien des zones de sensibilité totale aux organophosphorés et aux carbamates que des zones où les phénotypes résistants représentaient jusqu'à 98% de la population anophélienne. Dans l'ensemble, la résistance était plus localisée et de niveau plus faible que l'on ne pensait.

Grâce aux méthodes de microtitrage, on a identifié deux mécanismes de résistance: l'un faisait intervenir une élévation des estérases (estérases non spécifiques) et l'autre une insensibilité de l'acétylcholinestérase, le premier mécanisme (observé pour la première fois chez des anophélinés d'Amérique centrale) étant prédominant. Aucun de ces mécanismes n'a été décelé dans les régions non agricoles et où l'on n'emploie que très peu les organophosphorés ou les carbamates pour la lutte antipaludique. Les deux mécanismes étaient observables, mais rares, dans les régions relativement peu agricoles; en revanche, on observait une fréquence élevée des deux mécanismes de résistance dans toute la région d'agriculture intensive le long de la côte pacifique.

Les deux mécanismes de résistance sont donc étroitement associés aux zones d'agriculture intensive, qui sont également des zones de très forte densité de moustiques et d'emploi intensif d'insecticides en santé publique. D'autres études seront nécessaires pour déterminer le rôle relatif de l'application de pesticides dans l'agriculture et en santé publique en ce qui concerne la modulation des niveaux de résistance observés en un lieu donné et à un moment donné. La sélection de la résistance sous l'effet de pulvérisations à but sanitaire a été observée lors d'études sur le terrain d'*A. albimanus* en Haïti et d'*A. culicifacies* à Sri Lanka.

Les données obtenues au Guatemala sont d'un intérêt particulier sur le plan opérationnel car elles montrent que la distribution de la résistance est hétérogène. Tout d'abord, les deux mécanismes de résistance (élévation des estérases et insensibilité de l'acétylcholinestérase), à part leur association avec les zones agricoles, semblent sélectionnés indépendamment. Lorsque l'un d'eux est fréquent dans une région, l'autre ne l'est pas nécessairement, comme il ressort de leur incidence relative dans deux régions du pays. De plus, la résistance due à l'un ou l'autre mécanisme peut être extrêmement localisée, comme le montrent plusieurs exemples dans chaque cas. Enfin, on observe une association entre les types locaux d'emploi d'insecticides et les foyers de résistance.

Le titrage portant sur l'élévation des estérases permet de déceler les moustiques hétérozygotes et homozygotes pour la sensibilité ou la résistance aux insecticides. Il faut toutefois être très prudent lors de l'analyse de données isolées ne révélant qu'un génotype car la présence de copies multiples des gènes de résistance peut compliquer l'analyse, la résistance étant par ailleurs de type dominant simple. Les titrages portant sur l'insensibilité de l'acétylcholinestérase n'ont montré que peu de génotypes homozygotes résistants, ce qui était prévisible avec un niveau de résistance aussi bas.

Ces méthodes constituent une nouvelle technologie prometteuse pour la détection et l'évaluation de la résistance et devraient faciliter les décisions en matière de stratégie de lutte.

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