DETECTION OF ORGANOPHOSPHATE DETOXIFYING ESTERASES BY DOT-BLOT IMMUNOASSAY IN CULEX MOSQUITOES[1]

VALERIE BEYSSAT-ARNAOUTY,[2,3] CLAUDE MOUCHES,[4] GEORGE P. GEORGHIOU[3] AND NICOLE PASTEUR[2]

ABSTRACT. A dot-blot immunoassay, using antiserum raised against esterase B1 responsible for organophosphate (OP) resistance in *Culex quinquefasciatus*, was used to study different laboratory strains and field collections of this species, as well as of *Cx. pipiens*, *Cx. tarsalis*, *Anopheles albimanus* and *An. stephensi*. The frequency of esterase B1 positive individuals revealed by this test was in agreement with the data obtained by other methods, indicating the potential of this method for the detection and monitoring of resistance due to increased detoxification by esterases.

INTRODUCTION

In recent years, interest has been generated in biochemical test methods for detecting and monitoring insecticide resistance which are more specific than traditional bioassays. The major objective of such tests is the detection of specific resistance mechanisms in individual insects and the estimation of the frequency of resistance genes in populations.

Most biochemical approaches developed thus far aim at the detection or measurement of enzymatic reactions in homogenates of single insects and use electrophoretic analysis, filter paper tests or microtiter plate assays. They are concerned with the detection of organophosphate (OP) and carbamate resistance that is due to reduced sensitivity of acetylcholinesterase (Raymond et al. 1985, Hemingway et al. 1987, O. Dary et al., unpublished data, Brogdon et al. 1988) or to increased detoxication by highly active esterases in mosquitoes (Georghiou and Pasteur 1978, Brogdon et al. 1988, Hemingway et al. 1987, Rees et al. 1985), *Nephotettix cincticeps* and *Laodelphax striatellus* (Ozaki 1969), *Phorodon humili* (Beck and Büchi 1980), *Myzus persicae* (Needham and Sawicki 1971, Ffrench-Constant and Devonshire 1986) and other insects. In the genus *Culex*, several OP detoxifying esterases have been identified by electrophoresis, some of which may coexist in the same individual (Raymond et al. 1987, Magnin et al. 1988). In such cases, detection methods based on total esterase activity, such as the filter paper test (Pasteur and Georghiou 1981, 1989) or microtiter plate assays (Hemingway et al. 1987, Brogdon et al. 1988), provide information on the frequency of resistant individuals, but not on the nature of the esterases involved.

Recently, Mouchès et al. (1987) have shown that antisera raised against two esterase types (A and B) observed in OP-resistant *Culex* do not cross-react. We have, therefore, investigated the feasibility of using a dot-blot immunoassay to identify the type of detoxifying esterase present in single insects and, thus, to improve the specificity of the available resistance detection methodology.

MATERIALS AND METHODS

The study was conducted on seven strains or field collections of *Culex quinquefasciatus* Say, four of *Cx. pipiens* Linn., two of *Anopheles albimanus* Wied. and one each of *Cx. tarsalis* Coq. and *An. stephensi* Liston (Table 1). The esterase types present in each collection were determined by starch electrophoresis (Raymond et al. 1987).

For dot-blot immunoassay, two types of homogenates were used: single insect homogenates prepared in 1000 µl of 0.025 M imidazole-saline buffer at pH 7.4, or mass homogenates of 10 mosquitoes (5 males and 5 females) in 100 µl of buffer. Homogenates were made in 1.5-ml Eppendorf tubes and centrifuged for 1 min in a tabletop microfuge at 9,000 g. Dot-immunoblot assays were performed in three steps: (a) dot-blotting the homogenate on nitrocellulose membrane, (b) probing the esterase contained in the homogenate with rabbit esterase B1 antiserum and (c) revealing the esterase/anti-esterase B1 complex using a goat anti-rabbit serum conjugated to alkaline phosphatase. The rabbit esterase B1 antiserum had been prepared by Mouchès et al. (1987) against esterase B1 of the Tem-R strain. Blotting was done by depositing 5-µl volumes of each homogenate on a nitrocellulose

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[2] Institut des Sciences de l'Evolution (CNRS, UA 327), Université de Montpellier II, 34060 Montpellier, France.

[3] Department of Entomology, University of California, Riverside, CA 92521, USA.

[4] Station de Zoologie, I.N.R.A., B.P. 2078, 06606 Antibes, France. Present address: IBEAS (CNRS UA 168), Université de Pau, Campus Universitaire, 64000 Pau, France.

membrane (Bio-Rad Trans Blot). The membrane was then: (a) immersed for 10 min in a denaturing solution (1% SDS, 0.5% β-mercaptoethanol in distilled water), (b) transferred for 15 min in a fixing solution (10% acetic acid, 25% isopropanol), (c) rinsed with distilled water and (d) air-dried on Whatman filter paper. The membranes (referred to as "blots") were then either processed immediately or stored in plastic bags at 4°C for processing within two months.

For immunodetection (probing), the blots were: (a) incubated for 30 min in TS buffer (0.05 M Tris, 0.2 M sodium chloride, at pH 7.4) containing 3% of bovine serum albumin (Fraction IV, Sigma, St. Louis, MO), (b) transferred for 30–60 min (or overnight) into the esterase B1 antiserum solution (37.5 μl of esterase B1 antiserum in 7.5 ml of TS buffer containing bovine serum albumin), (c) washed three times for 10 min in TS buffer containing 0.2% Triton X-100 and 0.2% SDS, and two times for 10 min in TS buffer, (d) immersed for 30 min in a secondary antibody (1.4 μl of 1 mg/ml goat anti-rabbit alkaline phosphatase conjugate in 10 ml of TS buffer containing bovine serum albumin), (e) washed as described in (c), and (f) incubated in 5 ml of Tris-HCl buffer (0.1 M Tris, 0.1 M NaCl, 0.0005 M $MgCl_2$, adjusted at pH 9.5 with HCl) containing 16.5 μl of 5-bromo-4-chloroindoxyl phosphate and 33 μl of nitroblue tetrazolium salt to reveal alkaline phosphatase activity (Blake et al. 1984). The alkaline phosphatase reaction was stopped by immersing the blots in 0.005 M EDTA. Finally, the blots were air-dried and stored in plastic bags or in Saran Wrap®.

The intensity of the blots was read either visually or by using a densitometer (model RCX, Tobias Associates, Ivyland, PA).

RESULTS

Elaboration of dot-blot immunoassay test: A series of tests were first run to determine the optimum volume and dilution of homogenate deposit needed on nitrocellulose membranes to produce the best discrimination between mosquitoes possessing or lacking highly active esterases. These tests used mass homogenates of adults of strains S-Lab (lacking an esterase of high activity), S54 (esterase A1), Tem-R (esterase B1), SeLax (esterases A2, B2) and Poso-Est (esterases A3, B3). A 5-μl volume was judged the best, as it produces spots that were sufficiently large to be read on a densitometer, as well as by eye.

Homogenate dilutions from 10–10,000× per insect were then compared. No difference in staining intensity was observed between deposits of S-Lab and S54 at any of these dilutions, confirming the observations of Mouchès et al. (1987) that antiserum B1 does not cross-react with type A esterases. To obtain a staining intensity similar to that of the 100× dilution of S-Lab, homogenates of Tem-R had to be diluted 5000×, SeLax 2000× and Poso-Est 400× (Fig. 1). Thus, the dot-blot immunoassay can discriminate between mosquitoes possessing type B esterase of increased activity and those lacking such enzymes (i.e., those lacking highly active esterases type B or possessing highly active esterases type A only).

Presence of increased quantity of esterase B in field populations: Since S-Lab homogenates give only minimal reaction at dilution 1000×, this dilution was chosen for comparison of single adults from laboratory or field strains of various species (Table 1). An average of 28 insects were tested per strain (range 16–48), except for Tem-R, of which 143 insects were analyzed. Each blot included a small number (1–5) of S-Lab insects as reference. The results of visual interpretation of the blots are given in Table 2. As expected, no positive reaction was observed with S-Lab adults or with the *Anopheles* strains Gorgas, EPR and Mal-R, which lack an esterase B of high activity. Three field strains contained a mixture of insects giving positive and negative reactions (Naples II, Westchester and Berkeley), and six contained mosquitoes giving only positive reactions. The proportions of resistant mosquitoes revealed by these tests are not statistically different (5% level) from those obtained on the same strains by the use of starch gel electrophoresis to identify the presence and frequency of highly active esterases (Georghiou et al., unpublished data), with the exception of Naples II ($P = 0.05$).

In addition to the qualitative visual interpretation, the staining intensities of blots obtained with Tem-R, Berkeley and Simpson adults were also compared quantitatively by densitometry. The optical densities (ODs) of Tem-R and Simpson adults were always larger than those of the susceptible S-Lab on the same blot, whereas the

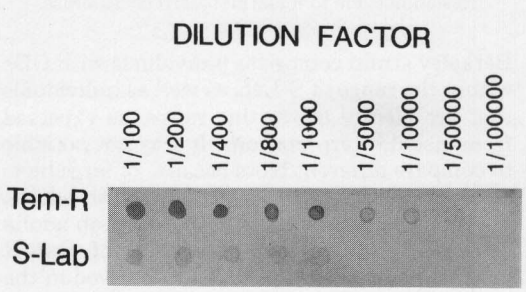

Fig. 1. Example of a blot comparing increasing dilutions of Tem-R and S-Lab mosquito homogenate. Note the differences in staining intensity at the various dilutions.

Table 1. Laboratory strains (*) and field collections (**) studied and types of highly active esterases they present.

Species/strain	Highly active esterase present	Origin	Reference
Culex quinquefasciatus			
S-Lab*	None[a]	California	Georghiou et al. (1966)
Tem-R*	B1	California	Ranasinghe and Georghiou (1979)
Naples II**	B1	Florida	Georghiou et al. (unpublished data)
SeLax*	A2 B2	California	Wirth et al. (1989)
Lahore**	A2 B2	Pakistan	Georghiou et al. (unpublished data)
Slidell**	B1, A2 B2	Louisiana	Georghiou et al. (unpublished data)
Houston**	B1, A2 B2	Texas	Georghiou et al. (unpublished data)
Culex pipiens			
Simpson**	B1	California	Georghiou et al. (unpublished data)
Berkeley**	B1	Illinois	Georghiou et al. (unpublished data)
Westchester**	B1	Illinois	Georghiou et al. (unpublished data)
S54*	A1	France	Pasteur and Sinègre (1978)
Culex tarsalis			
Poso-Est*	A3 B3	California	Georghiou et al. (unpublished data)
Anopheles albimanus			
Gorgas*	None[a]	Panama	Ayad and Georghiou (1975)
EPR*	None[b]	Guatemala	Georghiou (1975)
Anopheles stephensi			
Mal-R*	None[c]	Pakistan	Scott and Georghiou (1986)

[a] Susceptible strains.
[b] OP and carbamate resistance due to insensitive acetylcholinesterase.
[c] Resistance due to malathion carboxylesterase.

Berkeley strain comprises individuals with ODs within the range of S-Lab, as well as individuals that are clearly above this range, as expected from visual interpretations. It was not possible to compare different blots because of large blot-to-blot variations in the ODs of S-Lab adults. For example, the range of ODs of S-Lab adults used as control in the blot testing of Tem-R insects covers the range of ODs observed in the blot testing of Simpson adults. This emphasizes the importance of including on each blot mosquitoes that lack esterases, as reference, and shows that for precise quantification of esterase B in single mosquitoes, serial dilutions must be tested.

DISCUSSION

We have shown that dot-blot immunoassays can be used to detect, in single insects, the presence of increased quantities of type B esterases that confer resistance to a large number of organophosphate insecticides in *Culex* mosquitoes. Although quantification of esterases B could not be performed using a single dilution

Table 2. Proportions of mosquitoes giving positive reaction by dot-blot immunoassay and those showing the presence of a highly active esterase by electrophoresis.

Strains or field collections[a]	Esterase positive[b]		Antiserum B1 positive[c]		Chi-square[d]
	No.	%	No.	%	
S-Lab	N	0	0/40	0	–
Tem-R	N	100	143/143	100	–
Lahore	N	100	44/44	100	–
SeLax	N	100	48/48	100	–
Naples II	50	70	12/25	48	5.76 (*)
Slidell	50	100	36/36	100	–
Westchester	50	48	14/35	48	0 (NS)
Houston	50	98	25/25	100	0.51 (NS)
Simpson	50	100	20/20	100	–
Berkeley	50	46	8/19	68.4	2.37 (NS)
Gorgas		0	0/16	0	–
EPR		0	0/16	0	–
Mal-R		0	0/16	0	–

[a] See Table 1 for species name and strain origin.
[b] Identified by starch gel electrophoresis (Georghiou et al., unpublished data); N indicates large number (several hundreds).
[c] Identified by dot-blot immunoassay.
[d] NS = Not significant; * = significant at the 0.05 level.

of mosquito homogenate, this could be achieved by serial dilutions.

Our results with antiserum that was raised against esterase B1 of Tem-R mosquitoes are in agreement with those of Mouchès *et al.* (1987) in showing that this antiserum cross-reacts with esterases B2 of *Cx. quinquefasciatus* and B3 of *Cx. tarsalis*, but not with type A (i.e., A1, A2, A3). Since in many *Culex* populations esterases A and B coexist, it will be desirable to duplicate each blot for processing separately with specific antibodies for each esterase type.

Dot-blot immunoassays do not require expensive, specialized equipment and are thus more practical than electrophoresis in field surveys of resistance. Furthermore, the three-step procedure of the test (blotting, probing and revealing) permits deferring the second step for several hours or days so that it may be extremely useful where mosquitoes require processing shortly after collecting but must be sent to a distant laboratory for other serological testing.

Immunoassays based on principles similar to those of the dot-blot test presented here have been tentatively used in the detection of esterases conferring resistance (Devonshire and Moores 1984, Devonshire et al. 1986, Hemingway et al. 1986). Brown and Brogdon (1987) suggested that glutathione dependent DDT-dehydrochlorinase could be investigated in *Anopheles* species using mouse monoclonal antibody anti-porcine glutathione-S-transferase. It is expected that as enzymes conferring resistance are isolated and their serological relationships established, the utility of immunoassays as an insecticide resistance detection method will be extended. The simplicity and advantages of dot-blot immunoassay will then need to be considered.

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