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DIAGNOSTIC DOSE OF SYNERGIZED D-PHENOTHRIN FOR INSECTICIDE SUSCEPTIBILITY TESTING BY BOTTLE BIOASSAY

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ABSTRACT. The diagnostic dose of d-phenothrin synergized 1:1 with piperonyl butoxide for testing insecticide susceptibility of mosquitoes by bottle bioassay is reported for 2 mosquito species, *Culex quinquefasciatus* and *Ochlerotatus taeniorhynchus*. The diagnostic dose was defined as 2 times the 95% lethal concentration (LC_{95}). LC_{50}, LC_{90}, and LC_{95} were estimated by probit analysis of dose–response data. Procedures for diluting the commercial-grade off-the-shelf pesticide in acetone, treating the bottles, and calculating baseline data for insecticide-susceptible mosquito populations are described. The advantages and disadvantages of testing off-the-shelf commercial-grade pesticides that are maintained on premises by mosquito control programs, in contrast to using reagent-grade chemicals purchased from a chemical supply house, are also discussed. Data obtained by this method can be invaluable in making timely management decisions about the choice of pesticides in a control program.

KEY WORDS d-Phenothrin, ANVIL® 10+10, insecticide resistance, *Culex quinquefasciatus, Ochlerotatus taeniorhynchus*

INTRODUCTION

Since 1999, the diagnostic dose for monitoring insecticide susceptibility by bottle bioassay has been available for most organophosphate and pyrethroid pesticides currently used in Florida mosquito control programs (McAllister and Brogdon 1999). The diagnostic dose is the lowest dose of insecticide that provides 100% mortality over the shortest achievable time. It is a threshold value that can be used to discriminate between populations of mosquitoes susceptible to an insecticide and those that are resistant. Standardization of the diagnostic dose and the establishment of baseline data for susceptible populations facilitate tracking resistance, permitting rational management decisions concerning the use of pesticides (WHO 1963, 1970, 1981). The diagnostic dose is not the same as the 100% lethal concentration (LC_{100}). A recent publication of the World Health Organization (WHO) defined the diagnostic dose as 2 times the LC_{100} (WHO 1998). Because of statistical problems defining the LC_{100}, including the exceedingly large confidence limits at this value, we defined the diagnostic dose as 2 times the LC_{95}, a value we can estimate with fairly narrow confidence limits.

During September 2001, several Florida mosquito control programs requested assistance from the senior author in evaluating ANVIL® 10+10 ULV as a mosquito adulticide. At that time, there were no published data on the diagnostic dose for d-phenothrin, the active ingredient in ANVIL 10+10 ULV. We report here the diagnostic dose of synergized d-phenothrin for testing insecticide susceptibility of mosquitoes by bottle bioassay on the basis of dose–response data of 2 mosquito species, *Culex quinquefasciatus* Say and *Ochlerotatus taeniorhynchus* (Wiedemann).

Bioassay detection of insecticide resistance in adult mosquitoes has been based on a standard method recommended by the World Health Organization (WHO 1981, Brogdon and McAllister 1998a). Brogdon and McAllister (1998b) modified the WHO resistance test kit through the use of insecticide-coated glass bottles and solutions of standard-grade insecticides and synergists. The bottle bioassay is more suited than the WHO kit to the needs of field personnel because it asks a simpler question: "Will the insecticide at a concentration that gives 100% mortality for a susceptible population kill the test mosquitoes during the same time interval?"

The bottle bioassay has proven to be a simple, reliable, and inexpensive method of evaluating insecticide susceptibility that is highly appropriate for susceptibility testing by mosquito control programs. Moreover, time–mortality data from insecticide-treated bottles can be used as an indicator of an insect's response to a specific concentration of pesticide. Data obtained by this method can be invaluable in making timely management decisions about the choice of pesticides in a control program. A negative feature of time–mortality data is that no standardized, easy-to-use statistical analysis is appropriate (Robertson and Preisler 1991). For this reason, we estimated LC_{95} values and their confidence limits from dose–response data and used these estimates to determine a diagnostic dose.

MATERIALS AND METHODS

Dose–response experiments: ANVIL 10+10 ULV was obtained from Clarke Mosquito Control Products, Inc. (Roselle, IL) with a certificate of analysis. Lot number 0011011-6-1A contained

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Table 1. Output from probit procedure.

Species	*n*	Slope ± SEM	LC_{50} Concentration (μg/ml)	LC_{50} 95% CL	LC_{90} Concentration (μg/ml)	LC_{90} 95% CL	LC_{95} Concentration (μg/ml)	LC_{95} 95% CL
Culex quinquefasciatus[1]	623	1.53 ± 0.11	0.97	0.82, 1.16	6.69	5.12, 9.41	11.55	8.33, 17.64
Ochlerotatus taeniorhynchus[2]	707	1.65 ± 0.11	1.69	1.45, 1.97	10.06	7.79, 13.86	16.68	12.28, 24.59

[1] Goodness of fit test by Pearson chi-square = 4.897 (34 df, $P > 0.1$), ns.
[2] Goodness of fit test by Pearson chi-square = 1.909 (34 df, $P > 0.1$), ns.

9.91% (by weight) d-phenothrin and 9.94% (by weight) technical piperonyl butoxide, a synergist. Stock solutions were prepared by pipetting 1 ml of ANVIL 10+10 ULV into a 100-ml volumetric flask and adding reagent-grade ACS (American Chemical Society) acetone to the 100-ml mark. Test solutions were prepared by diluting the stock solution to yield solutions containing 0.08, 0.22, 0.88, 2.2, 8.8, 22, and 88 μg of active ingredient per 1 ml of ACS acetone. Bottle preparation followed the standard Centers for Disease Control and Prevention (CDC) protocol (Brogdon and McAllister 1998b, McAllister and Brogdon 1999). Test solution (1 ml) was added to each test bottle. We used 250-ml Wheaton bottles with fluorocarbon resin–lined caps (Wheaton Science Products, Millville, NJ). Controls consisted of bottles prepared with 1 ml of ACS acetone without any active ingredient. Fifteen to 20 female mosquitoes 3–5 days old were aspirated into each bottle. If male mosquitoes were accidentally introduced into the bottles, they were ignored in the analysis. Three bottles were prepared at each concentration for each experiment. Fresh test solutions were prepared for each of 3 replicate experiments. A battery-operated mechanical aspirator (Hausherr's Machine Works, Tom's River, NJ) was used to introduce mosquitoes into the bottles in order to minimize introduction of humidity that can occur with a mouth aspirator. Mosquito mortality was recorded at 1 h. A mosquito was recorded as dead if it was lying on its back or side and was unable to maintain flight after a gentle tap on the bottle. The resulting dose–response data were subjected to probit analysis (SAS Institute 2002). Dose–response data were fit to a log probit model by maximum likelihood procedures (Robertson and Preisler 1991) on the basis of the joint probability of all the observations (SAS Institute 2002).

Time–mortality experiments: Once the diagnostic dose was established from dose–mortality experiments, baseline response curves were compared by time–mortality experiments following the bottle bioassay methods of McAllister and Brogdon (1999). A stock solution was prepared as described for the dose–response experiments. Test solutions were prepared by diluting the stock solution with ACS acetone to yield the test concentrations indicated in Figs. 3–5. Controls consisted of 250-ml Wheaton bottles prepared with 1 ml of ACS acetone without any active ingredient. Time–mortality experiments were carried out in order to develop detailed protocols for the benefit of mosquito control programs. Time–mortality data, rather than dose–mortality data, are used by Florida mosquito control programs to evaluate susceptibility of target mosquito species.

Test mosquitoes: The mosquito species used in this study were *Cx. quinquefasciatus* Say and *Oc. taeniorhynchus* (Wiedemann). The *Cx. quinquefasciatus* were obtained from 2 laboratory colonies. One was a laboratory colony established in 1979 and maintained at Florida A&M University at the John A. Mulrennan, Sr., Public Health Entomology Research and Education Center (PHEREC), Panama City, FL. The 2nd was a laboratory colony maintained at the CDC in Atlanta, GA. The *Oc. taeniorhynchus* were from 2 sources. One field population was collected by CDC light trap at Feather Sound, Pinellas County, FL, on September 4, 2001. The other was a laboratory colony, the "Flamingo, Florida" strain, that has been maintained at PHEREC since 1983.

RESULTS

Dose–response experiments: Output from the probit procedure is shown in Table 1 and Figs. 1 and 2. The results of goodness of fit tests of the dose–response data to the statistical model are given as footnotes to Table 1. Because the chi-square values were not significant ($P > 0.1$), confidence limits were calculated with a *t*-value of 1.96 (Table 1).

The 95% confidence limits of LC_{90} and LC_{95} of both laboratory-reared mosquito species overlapped and therefore were not significantly different. The LC_{95} for *Cx. quinquefasciatus* was 11.55 μg/ml. Doubling this value gives a diagnostic dose of 23 μg/ml for this species. The LC_{95} for *Oc. taeniorhynchus* was 16.68 μg/ml. Doubling this value gives a diagnostic dose of 33 μg/ml for this species.

Time–mortality experiments: Time–mortality data from the laboratory-reared and field-collected mosquitoes are presented in Figs. 3–5. Figure 5 shows comparative baseline data for susceptible

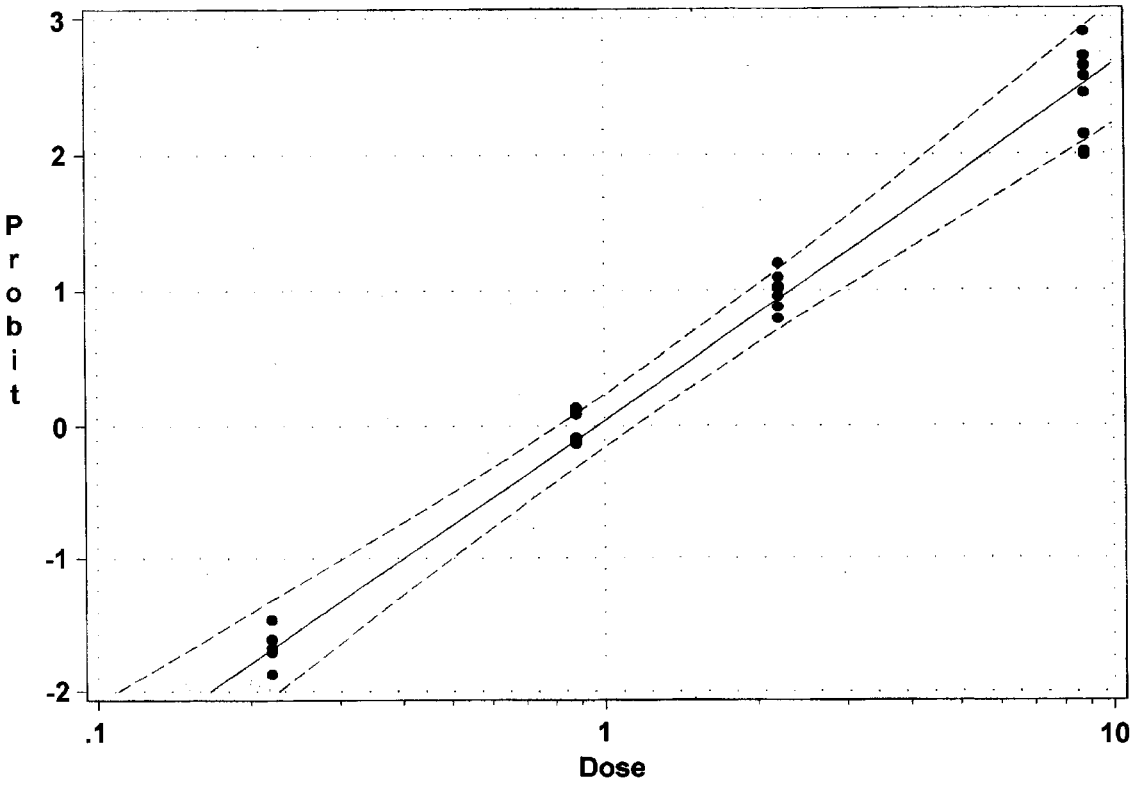

Fig. 1. Output from probit procedure for *Culex quinquefasciatus*. *x*-Axis, log dose (μg d-phenothrin/ml acetone); *y*-axis, probability units (probits). $n = 623$.

colonies of *Oc. taeniorhynchus* and *Cx. quinquefasciatus*.

DISCUSSION

Commercially available ANVIL 10+10 ULV was serially diluted with acetone to yield concentrations that were tested in dose–response experiments designed to determine the diagnostic dose. The use of synergized d-phenothrin was justified because our objective was to establish the diagnostic dose of an off-the-shelf commercial-grade insecticide that was readily available to mosquito control programs.

The diagnostic dose is useful in distinguishing susceptible insects from those that might show genetic resistance. The diagnostic dose is the minimum amount of active ingredient that kills the susceptible target species in the shortest achievable time. Test doses greater than this value could conceal lower levels of resistance, whereas doses less than this value might result in false reporting of resistance. Because of statistical difficulties in defining the LC_{100} (Robertson and Preisler 1991), we defined the diagnostic dose as 2 times the LC_{95}, a figure that can be statistically estimated by probit analysis with appropriate confidence limits.

The *Cx. quinquefasciatus* used in this study were from 2 laboratory colonies that have not been under insecticide selective pressure. Such mosquitoes are useful for establishing baseline data for comparison with field-collected mosquitoes. In contrast, the *Oc. taeniorhynchus* shown in Fig. 3 were field-collected as adults at Feather Sound, a mangrove swamp under periodic pyrethroid insecticide treatments. Different exposures might explain why the response curve of the latter field population extends to 90 min, whereas the *Oc. taeniorhynchus* from the susceptible colony were dead in 60 min (Fig. 5). The *Oc. taeniorhynchus* from Feather Sound were most probably under insecticide selective pressure.

This study contributes to resistance testing because, at present, the WHO insecticide test kit does not include treated test papers for d-phenothrin and there are no commercially available test papers for this pesticide. The only alternative, therefore, has been to formulate test solutions from available sources of d-phenothrin. The long-term objectives of the extension/outreach program of PHEREC are to establish diagnostic doses for mosquito insecticides used in the state of Florida, establish baseline data for specific species, and standardize methods of insecticide susceptibility testing throughout the state.

An advantage of the methods described here is that mosquito control programs can use the off-the-

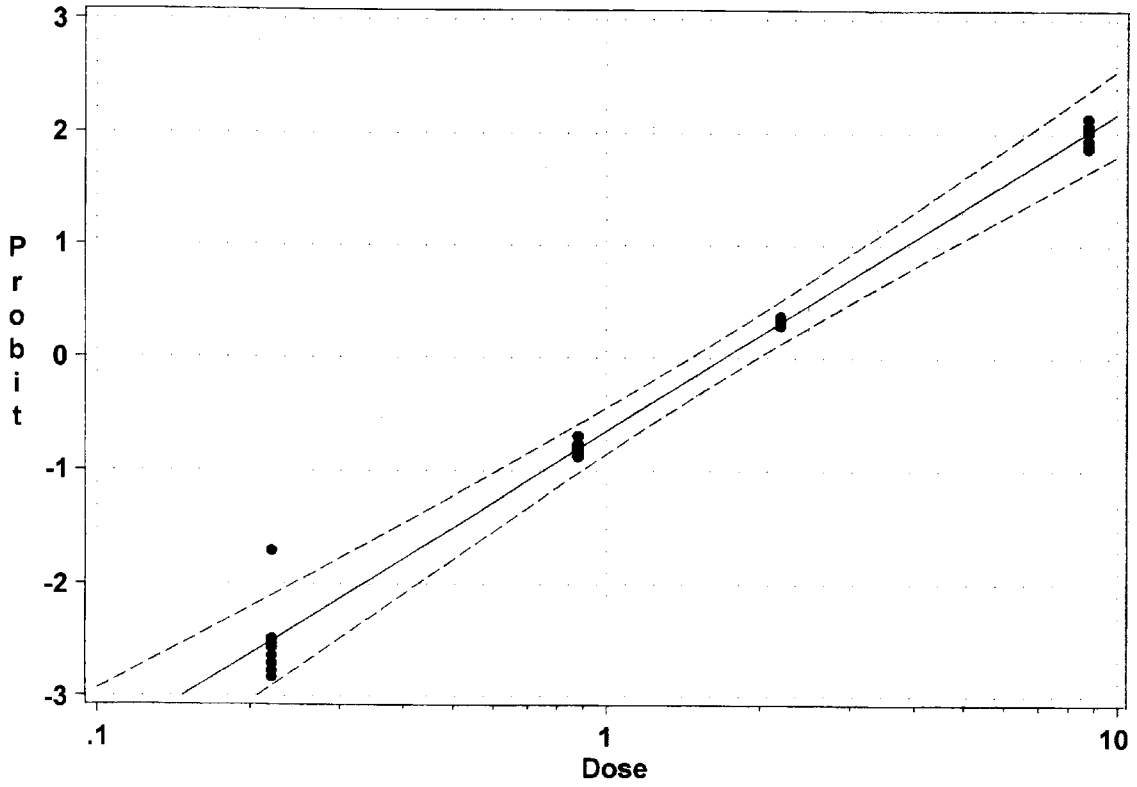

Fig. 2. Output from probit procedure for *Ochlerotatus taeniorhynchus*. *x*-Axis, log dose (μg d-phenothrin/ml acetone); *y*-axis, probability units (probits). $n = 707$.

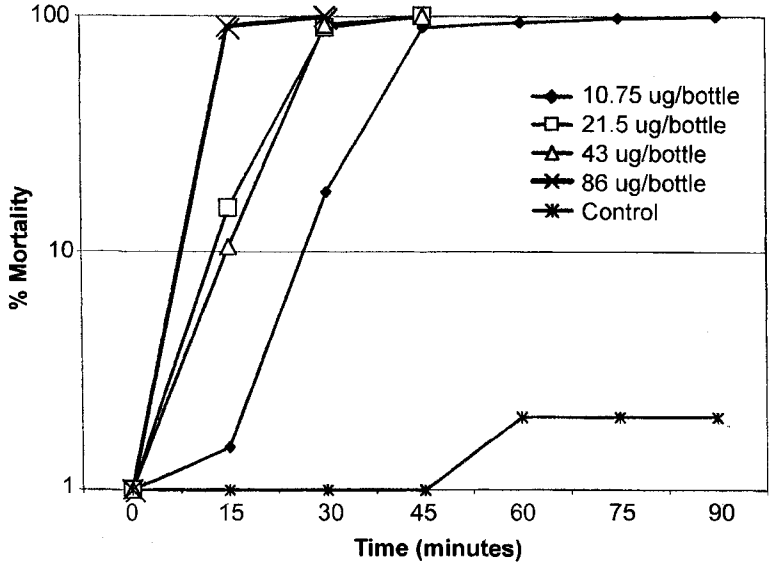

Fig. 3. Time–mortality data from bottle bioassay prepared with 4 concentrations of synergized d-phenothrin (1:1). Mosquito species *Ochlerotatus taeniorhynchus* was field-collected as adults at Feather Sound, Pinellas County, FL, on September 4, 2001, and tested on September 6, 2001. $n = 585$.

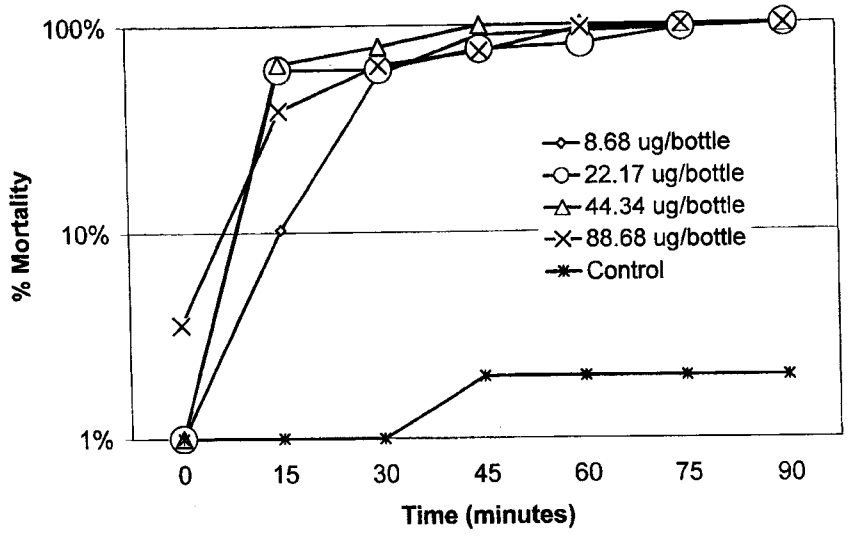

Fig. 4. Time–mortality data from bottle bioassay prepared with 4 concentrations of synergized d-phenothrin (1:1). Mosquito species *Culex quinquefasciatus* from CDC susceptible colony was tested on November 6, 2001. n = 236.

shelf pesticides that are used in their control programs and do not have to purchase reagent-grade chemicals from a chemical supply company. Baseline data from a susceptible population of mosquitoes establish the standard by which pesticide resistance is evaluated. This method is most valuable when testing is performed systematically at the beginning and at the end of each control season.

Another advantage of this procedure is that the active ingredient can be formulated with or without the addition of a synergist, an option not permitted by the WHO test kit. In the work reported here, the synergist piperonyl butoxide (PBO) was included in every test run.

A disadvantage of using pesticides that are maintained on premises is that storage conditions and degradation over time can introduce undesirable variations in results. A way to avoid this problem is to include a fresh standard for comparison. Standards for most adulticides currently used in Florida are available in test kits from PHEREC.

The diagnostic dose reported here for d-phenoth-

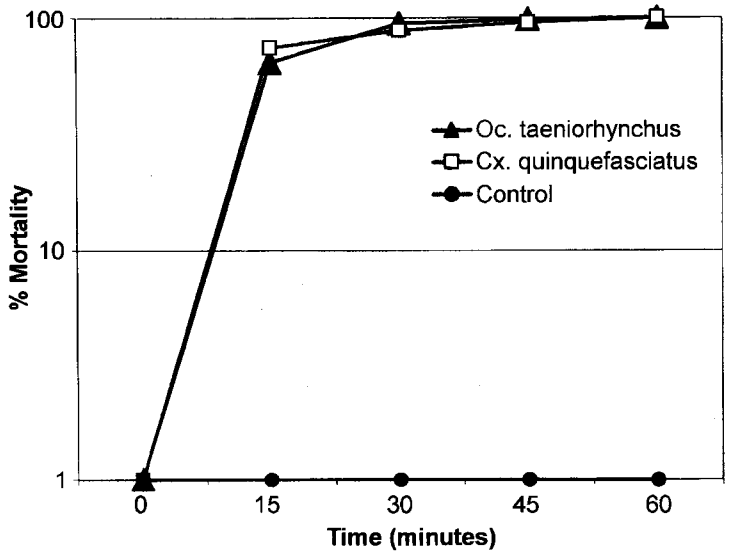

Fig. 5. Time–mortality data from bottle bioassay prepared with 22 μg of synergized d-phenothrin (1:1). Mosquito species are *Ochlerotatus taeniorhynchus* (n = 243) and *Culex quinquefasciatus* (n = 183), both from the PHEREC laboratory colony. These are the baseline data for these species.

rin applies only to the 2 species tested and might not be applicable to other species of public health importance. Baseline data for additional mosquito species are still needed and will be the focus of future work.

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