

# AN ALTERNATIVE BIOASSAY EMPLOYING NEONATE LARVAE FOR DETERMINING THE TOXICITY OF SUSPENDED PARTICLES TO MOSQUITOES

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**ABSTRACT.** An alternative bioassay using neonate larvae of *Aedes aegypti* is described for determining the toxicity of suspended particles, particularly insecticidal bacteria, to mosquitoes. This new assay is comparable in precision to the 4th instar bioassay recommended by the World Health Organization, but is quicker and requires much less space. Values obtained using neonate larvae as the test insect showed less variation within and between replicates in regard to  $LC_{50}$  and slope of the regression line, and in general required fewer replicates to fulfill the requirements for a statistically valid bioassay than the W.H.O. assay. The precision of the neonate larval bioassay in combination with its requirements for less time and space may make it especially useful for screening large numbers of samples.

## INTRODUCTION

The most precise method for measuring the potency of a microbial insecticide is the bioassay. As a result, bioassays are used routinely in experimental studies to determine toxicity, and in quality control programs to standardize the potency of industrial products. To determine the potency of an unknown preparation, the unknown is compared in parallel bioassays with a standard of known toxicity. The toxicity of the unknown is established on the basis of the ratio of the  $LC_{50}$ s (Dulmage 1981).

The development of *Bacillus thuringiensis* subsp. *israelensis* as a larvicide for mosquitoes and blackflies led to the proposal of two powders with arbitrarily defined potencies as standards for use in bioassays, and standardized protocols for conducting bioassays (World Health Organization 1981, McLaughlin et al. 1984). In both protocols the mosquito used to assay preparations is the 4th larval instar of *Aedes aegypti* (Linn.). Although use of this instar provides precise results, the preparation of the assay can be cumbersome, and requires considerable space, particularly if a series of assays is carried out simultaneously. To overcome these problems, we developed a bioassay in which the test suspensions are assayed in microtiter plates against neonate larvae of *Ae. aegypti*. In this paper we describe this bioassay, which is more rapid than the 4th instar bioassay, comparable in precision and requires much less space.

## MATERIALS AND METHODS

**Experimental design.** To determine the efficiency and precision of the alternative bioassay described below, a series of comparative bioassays was performed in which the new assay was compared with the one recommended by the World Health Organization (1981). A sufficient number of replicates was conducted on different

days until the data obtained fulfilled the following statistical requirements:

1. Chi-square value was lower than 5 (i.e., heterogeneity factor  $<1$ ).
2. Over a range of 6 concentrations tested per replicate, at least 2 concentrations were above, and 2 below the estimated  $LC_{50}$  (Concentration range equilibrium, CRE).
3. At least 4 concentrations resulted in mortality between 10 and 90% (Concentration range width, CRW).
4. Value of the slope was between 1.5 and 6.
5. Quotient between the high fiducial limit ( $P = 0.95$ ) and the low fiducial limit ( $P = 0.95$ ) was lower than 2.
6. At least 3 replicates were conducted, each on a different day.
7. Coefficient of variation of the  $LC_{50}$ s from the replicates was lower than 20%.
8. No pupation was obtained in the control.
9. Natural mortality in controls was  $\leq 10\%$ .

After these requirements were fulfilled, the mean  $LC_{50}$ s of the test material using each protocol were calculated using Probit analysis (Finney 1952). The efficiency and precision of each protocol was measured in terms of time required to determine the  $LC_{50}$ , and the precision of the  $LC_{50}$  was determined by variations in natural mortality, slope of the regression line, and chi-square values within and between bioassays.

**Test materials.** In order to avoid any preconception regarding the toxicity of the material used to compare the bioassay protocols, we first used the PG-14 isolate of *Bacillus thuringiensis* subsp. *morrisoni* obtained from Dr. L. Padua, National Institutes of Biotechnology and Applied Microbiology, University of the Philippines at Los Baños, The Philippines. The toxic activity of this new isolate has only received a preliminary characterization (Padua et al. 1984). We used nutrient broth to culture this isolate because parasporal body production on this medium is lower than in GYS (Glucose-Yeast-

Salts) broth (Konikkara 1984),<sup>1</sup> in terms of both the number of parasporal bodies produced per unit of medium, and their size. Thus, we artificially decreased the optimum toxicity level so that we could start with preliminary bioassays as if the test material had a completely unknown toxicity. This avoided any bias for or against one of the protocols. For fermentation, flasks were shaken at 340 rpm at 30°C for 72 hr or until autolysis was complete. The spore-parasporal body complex was pelleted and washed 3 times in double distilled water (Ibarra and Federici 1986). The dry weight of the materials in suspension was determined by drying 3 equal aliquots and weighing the dry material on a Cahn balance.

After we completed our comparison of the 2 protocols, we used the neonate bioassay to determine the toxicity of the standard assay preparation, IPS-82, of *B. thuringiensis* subsp. *israelensis*, which we obtained from Dr. H. deBarjac, Institut Pasteur, Paris, France.

**Mosquito colony maintenance.** A colony of *Ae. aegypti* was maintained at  $27 \pm 2^\circ\text{C}$  in a room with a 16:8 hr light:dark regime. Larvae were reared in  $33 \times 24$  cm enamel pans in dechlorinated tap water, and were fed 2–3 times daily with a 2:1 mixture of finely ground Gaines dog biscuit and wheat (Nutrex 540). Adults were provided with cut raisins as a source of sugars and water and were periodically supplied with mice for blood meals. Wet towels were placed in adult holding cages for oviposition and were removed every few days and allowed to dry slowly over a period of 48–72 hours. Neonate larvae used in the bioassays were obtained from eggs 1–3 weeks old.

**Bioassay protocol.** Concentrated test suspensions were diluted 1:100 in dechlorinated water, and then 2:100. Further dilutions were prepared according to the concentrations to be tested which ranged from 25 to 1.6 ng/ml in different serial dilutions of 6 concentrations each. Each series was adjusted according to previous results, if necessary. The suspensions were prepared in 25 ml dechlorinated water, pH 7 (neonate larvae died within a few hours in deionized water), each at a concentration of 0.75 of the previous suspension. Then each of the 4 troughs in the loading tray (see Fig. 1 for details) of a Vaccu-pette/96 multiple pipette tray® (Vanguard International, Inc., Neptune, NJ) was loaded with 25 ml of a different concentration of the test material. The test suspensions were then drawn into the Vaccu-pette multiple pipette by suction,

and the wells of a Corning® 96-well tissue culture plate (#25860) were loaded, 24 wells per concentration, with each of the test suspensions, 0.2 ml of the suspension per well. Thus, use of the Vaccu-pette/96 permitted all 96 wells to be loaded simultaneously. The assay of 6 concentrations plus a control required the use of 2 loading trays or the repeated use of a single tray. Markings on the syringe were used to calibrate the amount of suspension delivered to each well. Dechlorinated water was used as a control for the test suspensions.

After loading the wells with the test suspensions, eggs were transferred from the dried towel to white paper using a fine brush. Individual eggs were then picked up and transferred to each well using the device shown in Fig. 2. Once the eggs had been loaded into the wells, the plates were placed in a vacuum chamber for 15 min to induce hatching. The plates were examined under a dissecting microscope and wells in which the eggs had hatched were marked. The plates were then placed in a sealed plastic box, along with moist towels to prevent evaporation, and held in an incubator at 30°C for 24 hours. Mortality was determined at the end of the 24 hour period by counting the number of dead larvae in marked wells with the aid of a dissecting microscope.

In the bioassays with 4th instars, the protocol recommended by the World Health Organization (W.H.O.) (1981) was used. Mortality was determined 48 hours after the assays were initiated.

Data were analyzed by Probit analysis using an SAS program.

## RESULTS

The data obtained from the series of bioassays using each protocol in which the toxicity of *B. thuringiensis* subsp. *morrisoni* was determined are shown in Tables 1 and 2. The parameters and data used to estimate the variability both within and between bioassays are given for the neonate larval assay in Table 1, and for the standard W.H.O. assay in Table 2. As can be seen from a comparison of the data provided in these tables, the neonate larval bioassay required fewer replicates to obtain a coefficient of variation of <20% of the  $\text{LC}_{50}$ s. In the neonate larval assay, the first assay was a preliminary test to establish the range of concentrations for the subsequent, more precise bioassays. This assay was useful for determining the range of concentrations to be used in subsequent assays, but the data obtained from it were not used in the calculation of an  $\text{LC}_{50}$  because the chi-square value was greater than 5. The data obtained in the next 3 assays (2–4) fulfilled all statistical requirements (see Materials and Methods), and

<sup>1</sup> Konikkara, I. D. 1984. On the delta-endotoxin of *Bacillus thuringiensis* var. *israelensis*. Ph.D. dissertation. Swiss Federal Institute of Technology, Zurich. 111 pp.

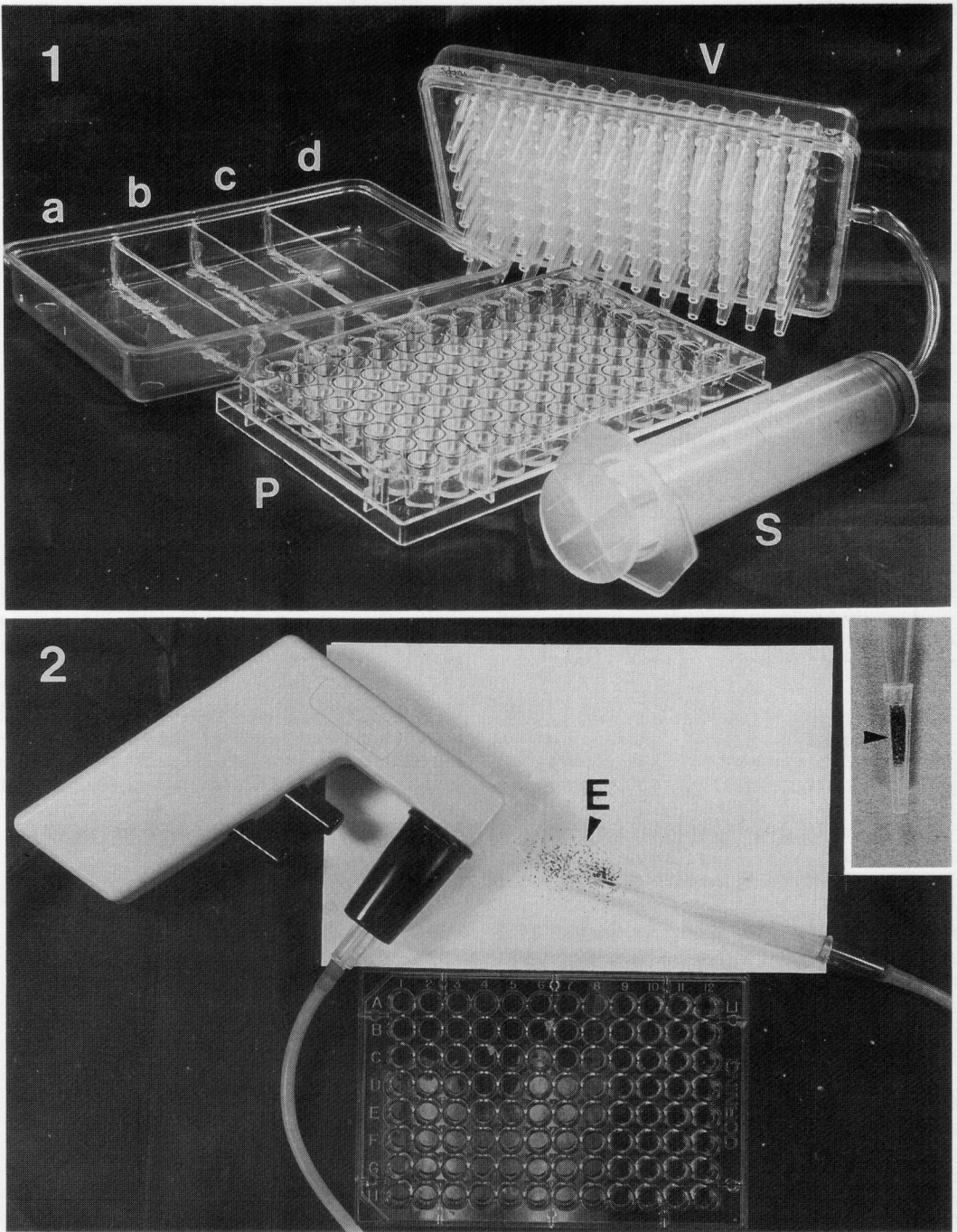


Fig. 1. Vaccu-pette and the 96-tissue culture plate system used in the neonate larval bioassay. Each of the 4 troughs (a-d) is loaded with a equal volume of a different concentration of the test suspension. The Vaccu-pette manifold (V) is then placed in the series of test suspensions and 0.2 ml of each concentration is drawn into each pipette tip simultaneously using the syringe (S). The manifold is then placed over the tissue culture plate and the 0.2 ml in each pipette tip is delivered to a corresponding well in the tissue culture plate (P).

Fig. 2. Apparatus used to transfer eggs of *Aedes aegypti* to the test suspension in the tissue culture plate wells. Embryonated eggs (E) are transferred with a brush from the paper on which they were oviposited to a piece of white paper. This separates the eggs from one another and makes them easy to see. Eggs are then picked up, one at a time, and transferred to the wells, one egg per well, using the automatic pipette illustrated. The pipette consists of a flexible rubber tubing (50 cm) with modified tip (inset) for handling the eggs. Each egg is drawn into the tip by depressing the suction trigger, and then released into the well with the delivery trigger. The tip (inset) consists of a 1 ml Eppendorf pipette tip over which is fitted the tip of a 0.2 ml pipette tip. A glass wool plug (arrow) at the end of the 1 ml pipette tip stops the egg from being drawn into the pipette.

Table 1. Statistical data obtained in bioassays using neonate larvae of *Aedes aegypti* to determine the toxicity of *Bacillus thuringiensis* subsp. *morrisoni* (PG-14).

| R | LC <sub>50</sub> | Max/Min | Slope | SE   | Chi-square | CRW | CRE | MLC <sub>50</sub> | SD   |
|---|------------------|---------|-------|------|------------|-----|-----|-------------------|------|
| 1 | 5.56             | 2.65*   | 3.33  | 0.91 | 6.47*      | ×   | ×   | —                 | —    |
| 2 | 3.55             | 1.55    | 3.51  | 0.56 | 3.09       | +   | +   | —                 | —    |
| 3 | 5.21             | 1.43    | 4.56  | 0.88 | 0.57       | +   | +   | —                 | —    |
| 4 | 4.12             | 1.46    | 3.40  | 0.63 | 3.04       | +   | +   | 4.29              | 0.84 |

R: Replicate number.

LC<sub>50</sub>: Lethal concentration at 50% mortality in ng/ml.

Max/Min: Fiducial limit quotient at P = 0.95.

SE: Standard error of the slope.

Chi-square: Chi-square value.

CRW: Concentration range width.

CRE: Concentration range equilibrium.

MLC<sub>50</sub>: Mean LC<sub>50</sub> from accepted replicates in ng/ml.

SD: Standard deviation of MLC<sub>50</sub>.

\*: Rejected value.

×: Failed test.

+: Passed test.

Table 2. Statistical data obtained in bioassays using 4th instar larvae of *Aedes aegypti* to determine the toxicity of *Bacillus thuringiensis* subsp. *morrisoni* (PG-14).

| R  | LC <sub>50</sub> | Max/Min | Slope | SE   | Chi-square | CRW | CRE | P | MLC <sub>50</sub> | SD   |
|----|------------------|---------|-------|------|------------|-----|-----|---|-------------------|------|
| 1  | NON-SIGNIFICANT  |         |       |      |            |     |     |   |                   |      |
| 2  | 35.10            | 1.68    | 5.39  | 1.47 | 2.55       | +   | ×   | + | —                 | —    |
| 3  | 40.88            | 1.67    | 4.42  | 1.10 | 0.78       | +   | +   | + | —                 | —    |
| 4  | 46.96            | 1.55    | 4.63  | 1.07 | 3.86       | +   | +   | + | —                 | —    |
| 5  | 47.46            | 1.49    | 5.14  | 1.18 | 7.39*      | +   | +   | × | —                 | —    |
| 6  | 45.66            | 1.75    | 3.57  | 0.95 | 4.79       | +   | +   | × | —                 | —    |
| 7  | 59.36            | 2.03*   | 3.07  | 0.93 | 3.10       | +   | +   | × | —                 | —    |
| 8  | 47.78            | 1.73    | 4.07  | 1.10 | 4.52       | +   | +   | × | —                 | —    |
| 9  | 35.91            | 1.47    | 5.77  | 1.33 | 1.34       | +   | +   | + | 41.25             | 5.53 |
| 10 | 40.07            | 1.54    | 4.61  | 1.06 | 0.79       | +   | +   | + | 40.96             | 4.56 |

NOTE: See explanation of abbreviations on Table 1.

P: Pupation of control larvae.

from these data we were able to estimate an LC<sub>50</sub> of  $4.29 \pm 0.84$  ng/ml with a coefficient of variation of 19%. The slope values were in the middle of the accepted range (1.5–6), and values for precision, as measured by the ratio of the upper and lower fiducial limits, were lower than 1.55.

When the standard W.H.O. bioassay using 4th instar larvae was employed, nine replicates were necessary to obtain a coefficient of variation of <20% for the estimated mean LC<sub>50</sub>. The comparatively larger number of replicates was necessary because the data from most did not fulfill all the basic requirements for an acceptable bioassay. There was no mortality in the first replicate, so a second test had to be carried out to establish the approximate range of concentrations to test. Results from this second replicate could not be used because they failed the concentration range equilibrium (CRE) requirement. The next 2 replicates were acceptable. However, when the third replicate necessary for estimating the mean LC<sub>50</sub> was carried out, this was rejected as well as the next 3 replicates because of pupation, high chi-square values, or

wide fiducial limits (5th and 7th replicates with a combination of these restrictions). Finally, by the 9th replicate, the data and requirements were sufficient to calculate a mean LC<sub>50</sub> of  $41.25 \pm 5.53$ , and an acceptable coefficient of variation of 13%. A 10th replicate was carried out as a further check of the estimated LC<sub>50</sub> to demonstrate that the LC<sub>50</sub> value was acceptable.

In general, the estimated LC<sub>50</sub>s obtained with the standard protocol showed little variation between replicates. However, the fiducial limits were usually wider than those obtained when neonate larvae were tested, with the highest quotient (within the acceptable limit) being 1.75. Slope values were also somewhat higher, but within the acceptable range, with the highest value being 5.77. The standard error of the slope (SE) was always higher than that obtained when neonate larvae were used. Chi-square values were similar for both techniques, except for the 5th replicate of the standard bioassay, which was rejected both because of its high chi-square value and pupation in the control.

The data obtained in the neonate larval bioas-

Table 3. Statistical data obtained in bioassays using neonate larvae of *Aedes aegypti* to determine the toxicity of the international standard IPS-82 of *Bacillus thuringiensis* subsp. *israelensis*.

| R | LC <sub>50</sub> | Max/Min | Slope | SE   | Chi-square | CRW | CRE | MLC <sub>50</sub> | SD   |
|---|------------------|---------|-------|------|------------|-----|-----|-------------------|------|
| 1 | 5.05             | 1.65    | 2.56  | 0.69 | 4.05       | +   | +   | —                 | —    |
| 2 | 4.71             | 1.67    | 2.39  | 0.66 | 1.38       | +   | +   | —                 | —    |
| 3 | 5.41             | 2.19*   | 1.57  | 0.61 | 3.17       | +   | +   | —                 | —    |
| 4 | 9.84             | 2.16*   | 1.85  | 0.37 | 2.74       | +   | +   | —                 | —    |
| 5 | 5.99             | 1.56    | 2.74  | 0.41 | 0.73       | +   | +   | —                 | —    |
| 6 | 5.21             | 1.66    | 2.57  | 0.41 | 3.06       | +   | +   | 5.25              | 0.54 |

NOTE: See explanation of abbreviations on Table 1.

says used to determine the toxicity of the standard preparation of *Bacillus thuringiensis* subsp. *israelensis* (IPS-82) are shown in Table 3. In this group of tests, six assays were required to estimate an LC<sub>50</sub> of  $5.24 \pm 0.54$  ng/ml for which the data fulfilled all the requirements for a statistically valid bioassay.

## DISCUSSION

In this group of tests, more replicates were required in general to estimate a valid mean LC<sub>50</sub> when 4th instars were used than when neonate larvae served as the test insect. This was a result of the need to reject many replicates using 4th instars because of high chi-square values, wide fiducial limits and/or the occurrence of pupation in the controls. The wider variations in data obtained with 4th instars is probably due to variations in size, weight and physiological age among larvae used in the assays. From a comparative standpoint, neonate larvae are physiologically more uniform, and therefore can be expected to provide a more uniform response when used as a test insect (Hughes and Wood 1981). First instar larvae also respond to lower concentrations of toxin and have been used previously by Davidson (1982) to assay the *B. sphaericus* toxin. The greater sensitivity of 1st instars accounts for the markedly lower LC<sub>50</sub> values in terms of ng/ml obtained with this stage in comparison to 4th instars. Nevertheless, the sources of variation noted above are probably not as important for experienced workers who can assess accurately the physiological age of 4th instars.

In the case of *B. thuringiensis* subsp. *israelensis* (IPS-82), although the number of assays required to estimate the LC<sub>50</sub> was greater than for *B. thuringiensis* subsp. *morrisoni* (6 versus 4), the precision of the results demonstrate the utility of using the neonate larval assay for determining the toxicity of other particulate materials.

Although the statistical data we have obtained indicate the neonate larval bioassay can be used to estimate LC<sub>50</sub>s with precision, perhaps the

most useful features of this assay are that it is quicker and requires much less space. The assay can be read in 24 hr, as compared to 48 hr for 4th instar assay, and the microtiter plates require much less than one-tenth the incubator space required to carry out the assays using 4th instars. The actual time for setting up the bioassay with neonate larvae is approximately 30 minutes longer due to the period required to induce hatching, and then marking the wells with eclosed larvae. However, the latter step can be eliminated if viable (uncollapsed) eggs are used, in which case about 95% of the larvae will eclose. Use of neonate larvae also avoids the tedious and possibly inaccurate selection of 4th instars of approximately the same age and physiological state. Other characteristics that make the neonate larval bioassay attractive are the ease of storing eggs, which makes continuous colony maintenance unnecessary, and elimination of the need to rear larvae to the 4th instar.

We have found the neonate larval bioassay to be useful in our studies, particularly in situations where a series of samples must be tested over a period of several days. We are not recommending that this bioassay replace the W.H.O. 4th instar assay, but rather that other investigators try the assay to determine its utility for their purposes.

## ACKNOWLEDGMENT

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