ARTICLES

THE WORLD HEALTH ORGANIZATION TEST KIT FOR DETECTION OF RESISTANCE IN MOSQUITO LARVAE

A. W. A. BROWN

Scientist (Biologist), World Health Organization, Geneva, Switzerland

Realizing the need for standard tests for resistance in insects of public health importance, the Expert Committee on Insecticides (1957) at its seventh session in July 1956 recommended the release of a trial method for mosquito larvae based on a fusion of existing techniques as summarrized by Brown (1957). Accordingly a tentative method was drawn up with the expert advice of Dr. J. R. Busvine and Dr. R. W. Fay, and 28 test kits were distributed in May 1957 to key workers throughout the world for a practical trial and suggestions for their improvement.

This tentative method involved exposing duplicate lots of 30 larvae in the third or early fourth instar to DDT, gamma-BHC or dieldrin at 5 concentrations ascending in multiples of 5; the larvae were exposed in 1 litre of water in enamel pans for 24 hours and removed to clean water for a further 24 hours. Meanwhile a paper had appeared by Jones (1957a) describing a sensitive method based on various periods of exposure to a standard concentration of 100 p.p.m. DDT and the determination of a median lethal time (MLT); this was similar to the principle utilized by Burchfield, Hilchey and Storrs (1952) of determining the period of exposure (T_{50}) causing 50 percent of the larvae to lose their negative phototropic response. Moreover Elliott (1957) was using a method based on 1-hour exposure to graded concentrations, followed by a 5-hour recovery period, to counteract the control mortality shown by sensitive species such as *Anopheles gambiae*.

The WHO tentative method was submitted to practical trial by Smith and Ford (Florida), Fay (Georgia), Lewallen (California), Blazquez (Venezuela), Floch and Fauran (French Guiana), Busvine (England), Gad (Jordan), Gramiccia (Iraq) and Armstrong and Bransby-Williams (Tanganyika). They reported favourably on the method and kit, while making certain suggestions for their improvement, and the results of the tests they made are available from the World Health Organization (1958). In addition, valuable comments were received from Gjullin (Oregon), Hawkins (Alabama), Bunn (Washington, D. C.) and Elliott (Nigeria). A most detailed and valuable series of evaluation tests was performed at the National Institutes of Health by Jones (1957); and the WHO tentative method was compared by Dews (1957) with the Orlando test method as currently employed by the Armed Forces.

The results gained during this trial period were reviewed in November 1957 by the Expert Committee on Insect Resistance and Vector Control (1958). Since it had been shown by Jones (1957b) that the size of the container had no effect on the LC_{50} levels in third instar larvae of *A. quadrimaculatus,* the volume of water recommended was reduced to 250 cc; since he had found the size of the test group to make no marked difference in the results, the number of larvae was reduced to 20–25 with an option of 10 in cases of scarcity. On the recommendation of the Orlando workers and the U. S. Army authorities, the containers were changed to glass vessels. Since transfer of the larvae to clean water for a second day greatly increased the control mortality, the

observation period was restricted to 1 day. Since the 24-hour control mortalities by this method were more frequently zero than not, even failing to exceed 12 percent with *A. gambiae,* it was considered that a shorter period of exposure should not be recommended for the standard method, although it might prove necessary for certain species of *Anopheles.*

The test kit weighs 2½ lbs. and measures 8 inches in length, 5 inches in width and 5½ inches in height (Fig. 1). The standard test method as drawn up by the Expert Committee on Insect Resistance and Vector Control is as follows:—

INSTRUCTIONS FOR DETERMINING THE SUSCEPTIBILITY OR RESISTANCE OF MOSQUITO LARVAE TO INSECTICIDES

Introduction

(a) In order to detect the emergence of an insecticide-resistant strain of a mosquito, it is necessary to have either basic data for the species before the wide use of insecticides or else to make comparisons with specimens from an untreated area. Where regular larvicide operations are undertaken to control mosquitoes, the normal susceptibility levels of the larvae should be determined as early as possible. To this end, several tests (a minimum of eight) should be performed at various localities and seasons, to assess normal biological variation.

(b) It is suggested that the tests be continued at regular intervals to determine any significant reduction in susceptibility. It appears that resistance may be suspected in mosquito larvae if there is an increase of 10–50 times over the original LC_{50}, or when a proportion of the population can no longer be killed by any of the available concentrations. From the comparatively small amount of data available at present, the indications are that when an LC_{50} for DDT in excess of 0.1 p.p.m. is found for *Aedes* or *Anopheles,* or an LC_{50} above 1 p.p.m. for *Culex,* resistance should be suspected.

(c) The previous history of the use of

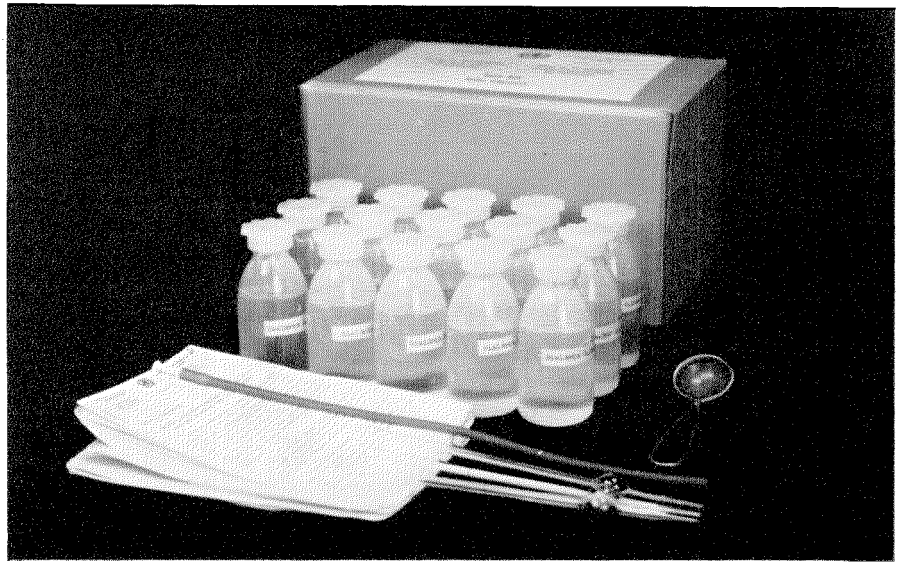

FIG. 1. WHO test kit

insecticides in the area, both in mosquito control and major agricultural uses, should be noted. It should be stressed that this test is not designed to indicate the relative effectiveness of the insecticides in the field where other formulations are used.

Composition of Test Kit

(a) Five different concentrations of each of three insecticides are provided, namely DDT (purified p,p' isomer), gamma-BHC or lindane (pure gamma isomer) and dieldrin (purified HEOD). They are in the form of 50-ml standard solutions in ethanol, which when added at the rate of 1 ml to 250 ml of water, give concentrations of 0.004, 0.02, 0.10, 0.50 and 2.50 p.p.m.; these concentrations are indicated on each bottle. A standard for 0.0008 p.p.m. dieldrin is also provided for very sensitive species such as certain anophelines.

(b) If it is desired to investigate additional intermediate concentrations, they may be prepared by diluting a portion of a standard solution with pure ethanol (e.g. a concentration of 0.01 p.p.m. may be obtained by diluting the 0.02 p.p.m. standard with an equal quantity of ethanol before taking the 1 ml for addition to the water in the vessel). If higher concentrations are required, they may be obtained from WHO on request.

(c) Three 1-ml pipettes are provided, one for each insecticide. A length of rubber tubing is included to act as a mouthpiece for the pipettes. Two eye-droppers and one metal strainer are also provided for transfer of the larvae. The user is expected to provide his own collecting and test vessels.

Method of Test

(a) For a complete test with one insecticide, sufficient larvae should be collected from the field in order that about 300 individuals of the same species may be selected; they should be in their third or early fourth instar and should be retained in the water in which they were collected until selection for testing. Any larvae showing abnormalities, for example a fuzzy appearance due to the presence of parasites on the body surface, should be discarded. Lots of 20–25 larvae are distributed in each of 12 small beakers each containing 25 ml of water. Their transfer is effected either by means of the strainer provided, or by means of an eye-dropper and a filter-paper cone; during the process they should be rinsed lightly in clean water.

(b) Into each of 12 glass vessels approximately 3–4 inches in diameter (jars, bowls or 500-ml beakers) add 225 ml of water. This water may be distilled, rain, well, stream or tap-water, as free as possible of chlorine or organic contaminants. It should be noted that distilled water obtained commercially may contain traces of poisonous heavy metals. Certain species, such as salt-marsh or tree-hole mosquitoes, may suffer on transfer to relatively pure water and this will be reflected in high control mortalities; in this case water from the breeding site should be used provided it is free of insecticides, care being exercised to exclude detritus. The average temperature of the water should be recorded and should be approximately 25° C; it must not be below 20° nor above 30° C.

(c) The test concentrations are now prepared by pipetting 1 ml of the appropriate standard insecticide solution under the surface of the water in each of the glass vessels and stirring vigorously for 30 seconds with the pipette. In preparing a series of concentrations, the most dilute should be prepared first. There should be two replicates at each concentration, and two control replicates.

(d) Within 15–30 minutes of the preparation of the test concentrations, the mosquito larvae are added to them by tipping the contents of the small beakers into the vessels.

(e) After a period of 24 hours, the numbers of (a) dead, (b) living and (c) moribund larvae are counted and recorded on the form provided. Dead larvae are those that cannot be induced to move when they are probed with a needle in the siphon or the cervical region. Moribund larvae are those incapable of rising to the surface

(within a reasonable period of time) or of showing the characteristic diving reaction when the water is disturbed; they may also show discoloration, unnatural positions, tremors, incoordination or rigor.

(f) As may be seen in the report form, larvae that pupate during the test are eliminated from consideration. Where more than 10 percent of the control larvae pupate in the course of the experiment, the test should be discarded. Tests with control mortality in excess of 20 percent are unsatisfactory and should be repeated.

(g) If the number of larvae available is limited, the test may be performed with only a total of 100 larvae; this involves 2 replicates of 10 larvae each at only 4 concentrations together with 2 controls. The range chosen should include one concentration expected to give complete kill.

General Remarks

(a) The accuracy of the concentrations provided will be affected if the alcohol is allowed to evaporate from the standard solutions. Therefore the bottles should be tightly stoppered after use. The contents should no longer be used when they have decreased below 5 ml. Fresh standard solutions should be obtained from WHO.

(b) Test vessels should be carefully cleaned after use to remove traces of insecticide. They should be thoroughly rinsed, scrubbed with detergent and water or cleaned with potassium dichromate solution, and rinsed again. Pipettes should be thoroughly cleaned with acetone or alcohol.

(c) In calculating the percentage mortalities for each concentration, the moribund and dead larvae in both replicates should be combined. The user may desire to construct the dosage-mortality regression line from the results obtained. For this purpose, logarithmic-probability paper has been provided, on which the results may be plotted. The regression line may be fitted by eye, and the LC_{50} or LC_{90} read from the graph. The regression line may be fitted more exactly by using the method of Litchfield and Wilcoxon (J. Pharm. Exp. Ther. *96*, 99, 1949), reprints of which may be obtained on request from WHO. Alternatively, the LC_{50} may be computed by the statistical method described in WHO/Mal/178 by Swaroop and Uemura.

(d) In cases where the control mortality is above 5 percent, the percentage mortalities should be corrected by Abbott's formula, viz:

$$\frac{\%\text{ Test mortality}-\%\text{ Control mortality}}{100-\%\text{ Control mortality}} \times 100$$

(e) The results recorded on the forms provided should be sent to the World Health Organization (Division of Environmental Sanitation), Palais des Nations, Geneva, Switzerland. Results with anophelines should be addressed to the Division of Malaria Eradication, WHO.

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