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WHO/Mal/199 ✓  
WHO/Insecticides/74  
18 February 1958

ORIGINAL: ENGLISH

ASSESSMENT OF SUSCEPTIBILITY TO INSECTICIDES  
IN MOSQUITO LARVAE

Summary of information received by the Divisions of Environmental  
Sanitation and of Malaria Eradication

The Expert Committee on Insecticides recommended in its Seventh Report (1956) the release of a trial method for mosquito larvae based on a fusion of existing techniques, as summarized by Brown (1957). The tentative method was drawn up accordingly, with the advice of Dr J. R. Busvine and Dr R. W. Fay, and 28 test kits were distributed to key personnel in various parts of the world. For each concentration of insecticide, the tentative method recommended duplicate tests with 30 larvae each in one litre of water in enamel pans.

The results of the tests performed by the various investigators are shown in Tables 1, 2 and 3. The percentage mortalities are based on the numbers completely dead after the 24 hours exposure period, the moribund being excluded from consideration. In determining the  $LC_{50}$  figure, the dosage-mortality regression line was fitted visually to the points given by the percentage mortalities corrected for control mortality by Abbott's formula.

In discussing the results, it is more intelligible to consider the  $LC_{50}$  values in units of parts per billion (i.e. parts per thousand million). As is well known, Anopheles larvae are normally very susceptible to DDT. The results show that the  $LC_{50}$  levels for A. albimanus and A. aquasalis are of the order of 5 p.p.b. or less. It is of interest to note that the laboratory colonies of A. atroparvus derived from a collection at Ferrara, Italy before DDT was ever used showed  $LC_{50}$ 's of 3.8 p.p.b. at Rome (E. Mosna, Laboratory Studies on the Development of Resistance to Insecticides in Anopheline Mosquitos. Mal. Sec. WHO Prog. Rep. 4, 1957). The results obtained with the Orlando laboratory strain in the 1957 tests obtained an  $LC_{50}$  value of 5 p.p.b., slightly higher than the  $LC_{50}$  of 1.5 p.p.b. reported for this strain seven years previously (Incho & Deonier, 1950), and of 2.5 p.p.b. 12 years ago (Deonier et al., 1945).

The results obtained with A. superpictus and A. sergenti in Jordan show  $LC_{50}$  values between 10 and 30 parts per billion. This is comparable to the value of 34 p.p.b. obtained for a laboratory stock of A. atroparvus originating in North Germany in 1948 (Kuhlow, 1957). Larvae of A. farauti at Hollandia, New Guinea, showed an  $LC_{50}$  of 30 p.p.b., some 4 months and 4 sprayings after a test which had showed the  $LC_{50}$  to be 5 p.p.b. (Davidson 1957a). The laboratory colony of A. quadrimaculatus at Bethesda was of this order of tolerance with an  $LC_{50}$  of 17 p.p.b. So also was the laboratory colony of A. gambiae at Arusha with an  $LC_{50}$  of 25 p.p.b.

Another point revealed by the use of the test kit on Anopheles larvae is the very high DDT-resistance in A. stephensi in Iraq. With  $LC_{50}$  values between 2000 and 3000 p.p.b., they are well in excess of the values obtained for a laboratory strain, which originated in Madras in 1938, of 540 p.p.b. at London (Davidson, 1957) and 340 p.p.b. at Hamburg (Kuhlow, 1957). This order of natural tolerance is shared by A. subpictus in unsprayed regions of India, the  $LC_{50}$  for a collection from Arthala being 250 p.p.b. (Sharma & Krishnamurthy). Field collections of A. quadrimaculatus in untreated areas in the Tennessee valley in 1950 showed an  $LC_{50}$  of approximately 110 p.p.b. (Ludvik, Snow & Hawkins, 1951).

Larvae of Culex are naturally much more tolerant to DDT than Anopheles larvae. The results obtained for C. fatigans at Cayenne show  $LC_{50}$  values as high as 150-250 p.p.b. These are substantially higher than those obtained previously: 25 p.p.b. at Lagos, Nigeria (Elliott, 1955), 40 p.p.b. at Maracay, Venezuela (Blazquez, 1957), 45 p.p.b. at Visalia, California (Gjullin & Peters, 1952), 52 p.p.b. at Clemwood, Rio Demarara (Elliott, communication to WHO) and 62 p.p.b. at Berkeley, California (Thevasagayam, 1957). In fact, the Cayenne population is resistant to gamma-BHC and dieldrin, and evidently the BHC pressure had induced some general vigor tolerance to DDT. But it should be noted that untouched Malayan strains at Tampin (Wharton, 1955) and Kuala Lumpur (Reid, 1955) showed  $LC_{50}$  levels of 145 and 224 p.p.b. respectively.

The value of 61 p.p.b. obtained for the  $LC_{50}$  of C. tarsalis at Kerman, California is considerably greater than that of 9 p.p.b. obtained for populations from untreated areas in Kern County, California in 1951 (Gjullin & Peters, 1952). Normal levels for C. pipiens in North America would appear to be between 13 p.p.b., as found at Princeton, New Jersey by Burbutis & Davis (1955) and 20 p.p.b. as found at Hamilton, Ontario by Brown, Armstrong & Peterson (1954).

Larvae of Aedes are normally very susceptible to DDT. For example, normal  $LC_{50}$  levels of A. aegypti range from 4 to 11 p.p.b. (Busvine, 1956), only an Indian strain showing  $LC_{50}$  figures up to 50 p.p.b. (Pal & Sharma, 1952). The figures of 34 to 80 p.p.b. for Aedes taeniorhynchus by the Orlando laboratory in 1957 therefore indicate an abnormal DDT-tolerance, especially when contrasted with the figure of 5 p.p.b. obtained at Titusville, Florida in 1949 (Deonier & Gilbert, 1950). The  $LC_{50}$  level of 350 p.p.b. found at Hanford, California in 1957 denotes an even higher DDT-resistance, especially when contrasted with the level of 11 p.p.b. found in untreated areas in Kern County in 1951 (Gjullin & Peters, 1952).

These tests give the first representative body of data on the susceptibility levels of the various species of Anopheles larvae to dieldrin. They show that the  $LC_{50}$  levels are below 4 parts per billion for A. albimanus, A. aquasalis, and even the DDT-resistant A. stephensi in Iraq. In this respect they resemble the Orlando laboratory colony of A. quadrimaculatus of 1950, which gave an  $LC_{50}$  of 0.8 p.p.b. dieldrin (Keller, Davis & Mooney, 1951). The results obtained with A. quadrimaculatus in 1957 show an  $LC_{50}$  of 25 p.p.b. for the Orlando colony; this heightened dieldrin-tolerance is also shown by the Arusha colony of A. gambiae.

The normal levels of Culex fatigans larvae to dieldrin have been determined as follows: 3 p.p.b. at Tampin, Malaya (Wharton, 1955), 5 p.p.b. at Lagos, Nigeria (Elliott, 1955), 6 p.p.b. at Kuala Lumpur, Malaya (Reid, 1955), and 16 p.p.b. at Berkeley, California (Thevasagayam, 1957). The  $LC_{50}$  level of 90-110 p.p.b. found at Cayenne therefore indicates a true dieldrin-resistance, although it was developed under BHC pressure; a similar instance of cross-resistance to dieldrin was described by Reid (1955) at Georgetown, Malaya, with an  $LC_{50}$  of 247 p.p.b.

Larvae of Aedes are evidently not so susceptible to dieldrin as are those of Anopheles or even Culex. The  $LC_{50}$  levels of normal strains of A. aegypti have been determined as follows: 14 p.p.b. at Kuala Lumpur (Wharton, 1955), 15 p.p.b. at Lagos (Elliott, communication to WHO), 40 p.p.b. at Carupano, Venezuela (Blazquez, 1957) and in a London laboratory strain (Shidrawi, 1957). The  $LC_{50}$  values of 8-20 p.p.b.

found in the 1957 tests with Aedes taeniorhynchus in Brevard County, Florida indicates a dieldrin-tolerance in excess of the 2 p.p.b. level found there in 1949 (Keller & McDuffie, 1952).

The only data previously available on the susceptibility of Anopheles larvae to gamma-BHC were obtained with A. quadrimaculatus. Incho & Deonier (1950) reported data indicating an  $LC_{50}$  of 5 p.p.b., and Keller, Davis & Mooney (1951) determined the  $LC_{50}$  at 13 p.p.b. The figures obtained for the same Orlando laboratory strain in 1957 were 30-75 p.p.b. Whereas the  $LC_{50}$  for A. aquasalis in French Guiana was less than 4 p.p.b., that for the DDT-resistant A. stephensi in Iraq was 80 p.p.b.

The normal susceptibility levels of Culex fatigans to gamma-BHC have been determined for three Malayan populations and the  $LC_{50}$  was found to lie between 14 and 26 p.p.b. The  $LC_{50}$  figures of 500-550 p.p.b. obtained at Cayenne with the test kit in 1957 indicate a significant BHC-resistance, greater than that recorded from Georgetown, Malaya (Reid, 1955), where the  $LC_{50}$  was 257 p.p.b.

No generalization can be made on larval BHC-susceptibility in the genus Aedes. The  $LC_{50}$  levels were 60 p.p.b. for a Kuala Lumpur strain (Wharton 1955), 90 p.p.b. for a strain from Klang, Malaya, and 140 p.p.b. for a London laboratory strain (Shidrawi, 1957).  $LC_{50}$  levels for untreated field populations of other species of Aedes are as follows: A. dorsalis in California, 26 p.p.b.; A. nigromaculis in California, 11 p.p.b. (Gjullin & Peters, 1952); and A. taeniorhynchus in Florida, 2 p.p.b. The  $LC_{50}$  figures of 18-50 p.p.b. obtained for A. taeniorhynchus in Florida in 1957 represent a significant increase in BHC-tolerance.

The text of the test method 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 mosquitos, 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 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 mosquitos, 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°C 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 discolouration, 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% of the control larvae pupate in the course of the experiment, the test should be discarded. Tests with control mortality in excess of 20% 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. 1949, 96, 99), 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%, 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.

TABLE 1. RESULTS OF TESTS FOR SUSCEPTIBILITY OF MOSQUITO LARVAE TO DDT

Species	Locality	Investigator	Date	Number	Control	Percentage mortalities at various p.p.m. of DDT					Corrected* MC50
						.004	.02	.10	.50	2.5	
<u>Anopheles superpictus</u>	Reseifeh, Jordan	A.M. Gad	19.IX.57	299	23	29	60	97	100	-	0.019
			26.IX.57	210	37	30	70	88	-	-	0.03
<u>Anopheles sergenti</u>	Fassayil, Jordan	A.M. Gad	30.XI.57	150	3	34	50	88	-	-	0.013
			28.XI.57	150	0	33	59	90	-	-	0.011
<u>Anopheles albimanus</u>	Maracay, Venezuela	J. Blazquez	Nov.57	538	18	60	71	100	100	100	0.004
<u>Anopheles aquasalis</u>	Cayenne, French Guiana	H. Floch & P. Fauran	12.X.57	330	3	90	100	100	100	-	<0.004
<u>Anopheles stephensi</u>	Om-el-Resas, Basrah, Iraq	G. Gramiccia	31.X.57	445	0	0	0	0	1	65	1.0
	Moawiya, Basrah, Iraq	G. Gramiccia	5.XI.57	197	0	-	-	0	10	-	>2.5
			8.XI.57	381	0	0	0	0	27	34	
<u>Culex fatigans</u>	Cayenne, French Guiana	H. Floch & P. Fauran	4.VII.57	358	2	0	0	29	98	100	0.15
			5.VII.57	356	0	2	3	7	88	100	0.25
<u>Aedes taeniorynchus</u>	Orlando, Florida	R. Ford	17.VII.57	300	0	7	17	53	88	-	0.08
			29.VII.57	300	0	22	30	88	98	-	0.034
<u>Aedes nigromaculis</u>	Hanford, California	L.L. Lewallen	7.X.57	360	23	15	25	28	77	98	0.35
<u>Culex tarsalis</u>	Kerman, California	L.L. Lewallen	7.IX.57	150	0	3	0	97	-	-	0.061
<u>Anopheles gambiae</u>	Arusha laboratory, Tanganyika	J.A. Armstrong	20.VIII.57	343	0	15	40	87	100	-	0.023
<u>Anopheles quadrimaculatus</u>	Orlando laboratory, Florida	R. Ford	7.VIII.57	300	0	33	100	100	100	-	0.005
	Bethesda laboratory, Maryland	J.C. Jones	7.X.57	300	0	30	100	100	100	-	0.005
			14.V.57	300	0	2	45	100	100	-	0.017
			15.V.57	300	0	3	30	100	100	-	0.017

\* Derived from dosage-mortality regression line obtained from mortalities corrected by Abbott's formula.  
/ At 1.0 p.p.m. DDT

TABLE 2. RESULTS OF TESTS FOR SUSCEPTIBILITY OF MOSQUITO LARVAE TO DIELDRIN

Species	Locality	Investigator	Date	Number	Control	Percentage mortalities at various ppm. of dieldrin						Corrected LC50
						.004	.02	.10	.50	2.5		
<u>Anopheles albimanus</u>	Maracay, Venezuela	J. Blazquez	Nov.57	283	33	83	100	100	100	-	-	<0.004
<u>Anopheles stephensi</u>	Om-el-Resas, Basrah, Iraq	G. Gramiccia	1.XI.57	315	0	87	100	100	100	-	-	<0.004
<u>Anopheles aquasalis</u>	Cayenne, French Guiana	H. Floch & P. Fauran	3.X.57	180	3	93	100	-	-	-	-	<0.004
<u>Anopheles quadrimaculatus</u>	Orlando laboratory, Florida	R. Ford	7.VIII.57 7.X.57	300 300	0 0	20 20	33 28	90 68	98 100	-	-	0.025 0.025
<u>Culex fatigans</u>	Cayenne, French Guiana	H. Floch & P. Fauran	13.VII.57 15.VII.57	300 300	0 0	5 0	5 2	15 15	95 88	-	-	0.09 0.11
<u>Anopheles gambiae</u>	Arusha laboratory, Tanganyika	W.R. Bransby-Williams	21.V.57 9.X.57	277 286	12 11	27 10	58 47	72 88	100 98	-	-	0.02 0.025
<u>Aedes taeniorhynchus</u>	Orlando, Florida	R. Ford	17.VII.57 29.VII.57	300 300	0 0	18 43	68 70	75 100	97 100	-	-	0.020 0.008

TABLE 3. RESULTS OF TESTS FOR SUSCEPTIBILITY OF MOSQUITO LARVAE TO GAMMA-EHC

Species	Locality	Investigator	Date	Number	Control	Percentage mortalities at various p.p.m. of Gamma-EHC					Corrected LC50
						.004	.02	.10	.50	2.5	
<u>Anopheles aquasalis</u>	Cayenne, French Guiana	H. Floch & P. Fauran	3.X.57	180	3	88	95	-	-	-	<0.004
<u>Anopheles albimanus</u>	Maracay, Venezuela	J. Blazquez	Nov.57	247	71	63	92	95	-	100	-
<u>Anopheles stephensi</u>	Om-el-Resas, Basrah, Iraq	G. Gramiccio	1.XI.57	348	0	0	2	71	100	-	0.08
	Moawiya, Basrah, Iraq		6.XI.57	306	0	0	0	90	100	-	0.08
<u>Anopheles quadrimaculatus</u>	Orlando laboratory Florida	R. Ford	7.VIII.57	300	0	5	5	63	100	-	0.075
			7.X.57	300	5	18	22	88	100	-	0.03
<u>Aedes taeniorhynchus</u>	Orlando, Florida	R. Ford	17.VII.57	300	0	0	15	88	100	-	0.05
			29.VII.57	300	0	8	60	92	100	-	0.018
<u>Culex fatigans</u>	Cayenne, French Guiana	H. Floch & P. Fauran	9.VII.57	360	5	15	12	25	35	98	0.5
			11.VII.57	361	0	2	2	28	37	97	0.55

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