Appendix

Appendix I Instructions for determining the susceptibility or resistance of adult mosquitoes. (WHO, 1981a)

1. Introduction

The purpose of the susceptibility test is to detect the presence of resistance individuals in an insect population as soon as possible so that alternative control plans can be made in time to deal with the situation when the insecticide in question is no longer having the desired effect.

When originally investigating an insect population two approaches are necessary:

(a) The establishment of the base-line susceptibility of a normal population. By "normal" is mean a population never subjected to insecticide pressure and in which resistant individuals are rare. Exposure of such a population to serial concentrations of insecticide or serial time exposures to a single insecticide concentration should yield a straight-line relationship between the log of the concentration or time and probit analysis. This is the discriminating or diagnostic concentration or time. From such data it is possible to predict by extrapolation that concentration which will normally kill all the individuals of a susceptible population. This is the discriminating or diagnostic concentration or time.

(b) The frequent exposure of a population under the insecticide selection pressure to this diagnostic concentration or time should serve to detect the appearance of abnormally tolerant individuals and to monitor changes their frequency.

1.1 Established diagnostic concentrations/ exposure times

Tentative diagnostic concentrations/ exposure times for adult mosquitoes are shown in Table 11. These data were obtained with unfed female mosquitoes at 27°c. It is likely that they will also apply to blood-fed specimens, though not for those entering hibernation. Since temperature affects the mortality, records if temperature should be made during the test. In general, higher temperature induces higher mortality and lower

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temperature results in higher survival. Therefore, the temperature/ toxicity correlation should be taken into account when performing successive tests under different temperature conditions.

Table 11 Tentative diagnostic concentrations and exposure times for adult mosquitoes.

Insecticides	*Anopheles spp.*		*Culex quiquefasciatus*	
	Concentration (%)	Time (hour)	Concentration (%)	Time (hour)
DDT	4	1	4	4
Dieldrin	0.4	1	4	1
Malathion	5	1	5	1
Fenitrothion	1	2	1	2
Propoxur	0.1	1	0.1	2
Permethrin	0.75	1	0.25	3
Deltamethrin	0.05	1	0.025	1
Lambdacyhalothrin	0.1	1	-	-

1.2 Use of diagnostic concentration/ exposure time

Tests should be made periodically with at least 75 mosquitoes and preferably with 100. It is recognized, however, that it may be difficult to obtain sufficient numbers to satisfy statistical requirements, especially when the insecticide is effectively reducing numbers, or in seasons when breeding is reduced.

A warning of possible incidence of resistance is given when survivors regularly appear in tests with a correctly selected diagnostic exposure. Occasional survivors in such checks could be due to normal variation. But the regular occurrence of survivors in three successive tests constitutes a warning signal calling for further investigation.

1.3 Condition of mosquitoes

Although there is seldom a large difference in susceptibility between the sexes, female mosquitoes (preferably blood-fed) should be used exclusively in field tests. This is because they survive better and show lower control mortality.

If mosquitoes are scarce, it is permissible to use a mixture of fed and unfed females, provided the proportion of each is recorded. Information about mosquitoes should be recorded on the formed provided.

1.4 Conditions of test

If possible, the experiment should be carried out indoors, in buildings free from insecticidal contamination and extremes of temperature, humidity, illumination and wind. Since temperature affects the mortality, temperatures should be recorded during the test. Also transport of mosquitoes to a base laboratory often results in mortality from causes other than the insecticides, this will show up as high mortality in the controls.

2. Composition of the test kit (Figure 10)

2.1 Equipment

2.1.1 Eight plastic tubes, 125 mm in length and 44 mm in diameter: 2 of which (with red dot) are used to expose the mosquitoes to the insecticides, 2 (with green dot) are used for the control exposure without insecticide and 4 (with green dot) are used as holding tubes for the pre-test sorting and post-exposure observation. Each tube is fitted at one end with 16-mesh screen.

2.1.2 Four slide units, each with a screw-cap on either side and provided with 20 mm filling hole.

2.1.3 Sixteen sheets of clean paper (12 x 15 cm) for lining the holding tubes.

2.1.4 Eight spring wire clips to hold the papers in position against the wall of the tubes. The 6 steel clips should be used only for the holding tubes and the control exposure tubes: the 2 copper clips should be used for the insecticide exposure tubes.

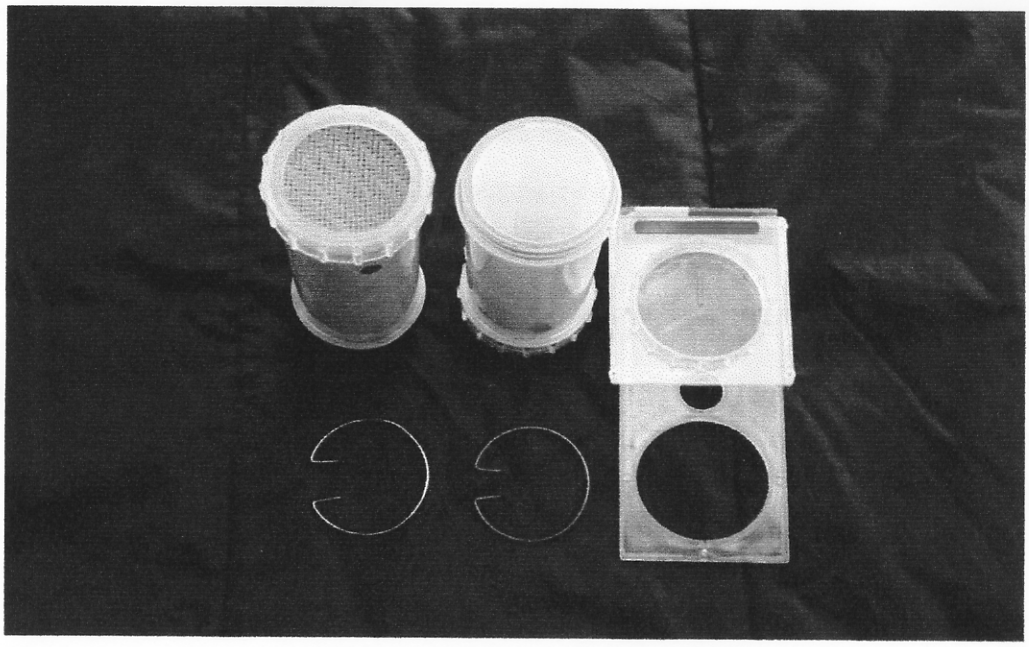


Figure 10 Composition of the test kit

2.1.5 Two glass aspirator tubes 12 mm internal diameter, together with 60 cm of tubing and mouthpieces.

2.1.6 One roll of self-adhesive plastic tape.

2.1.7 Instruction sheets and 20 report forms, plus 3 sheets of log probability paper for plotting regression lines using variable times with one concentration.

3. Test procedures

3.1 Into each of the holding tubes, insert a piece of clean white paper rolled into a cylinder to line the wall and fasten it in position with a spring-wire clip (silver). Attach the slides to the tubes.

3.2 Collect female mosquitoes with the aspirator (Figure 11A) provided to the holding cup (Figure 11B) about 25 per cup.

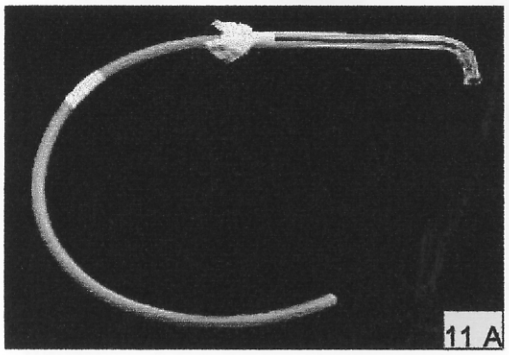
3.3 A pre-test holding period may be necessary to guard against including damaged specimens in the test. For this purpose, the holding cups are set upright, screen end up, for 1 hr. At the end of this time the damaged insects are removed.

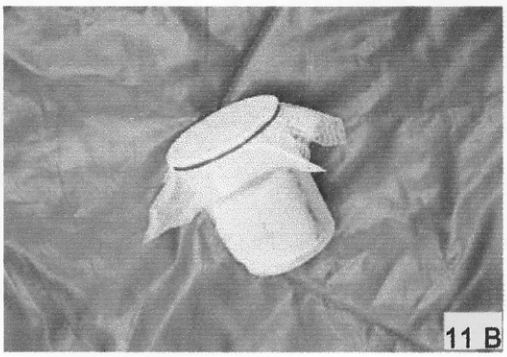
3.4 Into each of the exposure tubes introduce a sheet of impregnated paper, rolled into a cylinder to line the wall and fastened into position with an appropriate spring-wire clip.

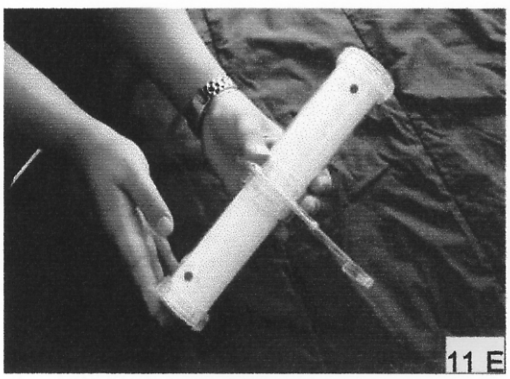
3.5 Introduce the mosquitoes into the exposure tube by aspirator though the filling-hole in each slide (Figure 11C). Close the slide.

3.6 Leave the exposure tube standing upright with screen end up for the required exposure period (Figure 11D) under conditions of moderate, diffuse illumination, and adequate humidity.

3.7 At the end of the required exposure period, transfer the mosquitoes to the holding tubes by attaching it to the vacant screw-top in the slide (Figure 11E). When some mosquitoes have been knocked down in the course of an exposure, the exposure tubes should be held horizontally and tapped to dislodge the insects from the slide before the latter is withdrawn. Attach the holding tube, open the slide and gently blow the mosquitoes into the holding tube: close the slide and remove the exposure tube. Then set the holding tube so that it stands on the slide and place a pad of moist cotton-wool on the screen (Figure 11F). Cardboard cartons or cups or other suitable containers may be used instead of the holding tubes, provided that are used consistently.







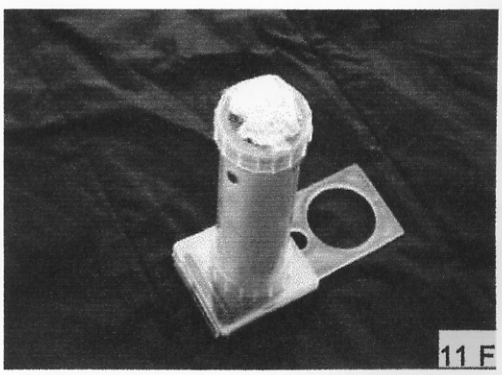


Figure 11 Method for determining the susceptibility or resistance of adult mosquitoes.

3.8 Keep the holding tubes for 24 h in a secluded, shaded place, where the temperature dose not exceed 30°c. Wherever feasible, the maximum and minimum temperature of the site of the holding tubes should be recorded. If necessary, the tubes should be protected from ants by placing them on a platform standing in a pan of water. If conditions are very hot and dry, a moist chamber may be prepared by suspending damp toweling in a container, and measuring the maximum and minimum temperature within.

3.9 Mortality counts are made after 24 h. Remove the dead mosquitoes by gently detaching the slide and cautiously moving the tube aside. Affected specimens that are unable to walk should be counted as dead. As an aid to counting the living specimens, they are stunned by a sharp jerk of the tube or stupify by chloroform or ether. The anaesthetics should not be allowed to come into direct contact with the plastic tube and screw-cap, which are soluble in these compounds. The results should be recorded on the forms provided.

3.10 Four replicate tests should be done with each of the diagnostic concentration and, preferably, 4 controls with oil-treated papers.

3.11 Tests with control mortality in excess of 20%, though unsatisfactory, should be recorded. An investigation into the causes of control mortality should be made and steps taken to avoid it. A possible cause may be the collection of mosquitoes from sprayed dwellings. In this case, it may be necessary to collect specimens from unsprayed ones, or to test adults reared from aquatic stages.

4. General remarks

4.1 Each impregnated paper may be used up to 20 times, and up to 3 weeks after removal from the package, provided all possible precautions are taken against evaporation of the insecticide solution. Organochlorine papers can be left in the tubes, with the open end well wrapped, and placed in the kit box, which in turn should be kept in a cool place. No paper should be used more than 3 weeks after removal from the package.

4.2 After an impregnated paper has been removed, the package should be resealed carefully with the plastic tape provided. The packages should be kept in a cool place, but not in a refrigerator, as too low a temperature may cause crystallization in the higher insecticidal concentrations. Prolonged storage at high temperatures should be avoided. Paper should not be used after the expiry date shown on the box. The expiry date is valid only if the packages are kept sealed at all times.

5. Interpretation of the results of the susceptibility tests

For the interpretation of the significance of the susceptibility tests the following aspects should be taken into account:

5.1 Physiological condition of insects, unfed, fed, gravid (and age when using in laboratory bred mosquitoes).

5.2 Place of collection. If the mosquitoes were collected from sprayed or unsprayed premises in a sprayed or unsprayed area.

5.3 Microclimatic conditions. Temperature and percentage of humidity during the exposure periods and during observational periods.

5.4 A slight decrease in susceptibility might be due to seasonal variations.

5.5 Number tested (whether large enough to be significant).

5.6 Mortality in controls.

5.7 Feeding status and type of food.

6. Processing and interpretation of results

The 24 hour mortality of mosquitoes exposed to standard concentration of insecticides should be plotted on logarithmic probability paper to construct the dosage mortality regression line or to construct the time mortality regression line when different batches of mosquitoes are exposed to the same concentration of insecticide, but for different periods of time (60 min, 120 min, 180 min) or shorter intervals depending on the standard concentration of insecticides used.

The following steps are performed;

6.1 Read the mortality at 24 hour for each test and calculate in percentages. If mortality in the control groups is over 5% but less than 20% a correction of mortality is made by applying the Abbot formula;

$$\text{corrected \% mortality} = \frac{\text{\% test mortality - \% control mortality}}{\text{100 - \% control mortality}} \times 100$$

If the mortality in controls is over 20% then tests are discarded.

6.2 Calculate an average of the mortality obtained to the same concentration in different triplicates.

6.3 Construct a regression line. If the regression line shows a drift to the right but remains parallel with the baseline data, this indicates an increase in tolerance only. If the regression line has a tendency to from a plateau at the upper part, this indicate resistance.

7 Criteria for assessing resistance status.

Insecticide susceptibility/resistance status is generally measured using the standard WHO susceptibility test procedures. In the use of diagnostic dosages, resistant status had been classified on criteria indicated below;

99-100% mortality	=	susceptible
80-98% mortality	=	verification reguired
< 80% mortality	=	resistant individuals present

Appendix II Preparation of diluted albumin (BSA) standards

Dilute the contents of one albumin standard (BSA) ampule into several clean vials, preferably in the same diluent as the samples. Each 1 ml ampule of albumin standard is sufficient to prepare a set of diluted standard for either working range suggested in Table 12. There will be sufficient volume for three replications of each diluted standard.

Table 12 Preparation of diluted albumin (BSA) standards

Vial	Volume of diluent (μl)	Volume and source of BSA	Final BSA concentration
A*	0	300 μl of stock	2,000 μg/ml
B*	125	375 μl of stock	1,500 μg/ml
C*	325	325 μl of stock	1,000 μg/ml
D*	175	175 μl of vial B dilution	750 μg/ml
E*	325	325 μl of vial C dilution	500 μg/ml
F*	325	325 μl of vial E dilution	250 μg/ml
G*	325	325 μl of vial F dilution	125 μg/ml
H*	400	100 μl of vial G dilution	25 μg/ml
I*	400	0	0 μg/ml = blank
A**	2,370	30 μl of stock	25 μg/ml
B**	4,950	50 μl of stock	20 μg/ml
C**	3,970	30 μl of stock	15 μg/ml
D**	2,500	2,500 μl of vial B dilution	10 μg/ml
E**	2,000	2,000 μl of vial B dilution	5 μg/ml
F**	1,500	1,500 μl of vial B dilution	2.5 μg/ml
G**	5,000	0	0 μg/ml = blank

* Dilution scheme for standard test tube and microplate protocols (working rang = 100 – 1,500 μg/ml)

** Dilution scheme for micro test tube or microplate protocols (working rang =1–25 μg /ml)

Appendix III Method to determine the protein concentration of individual mosquito.

The average 595 nm reading for the blank replications was subtracted from 595 nm readings of all other individual standard and unknown sample replicates. Standard curve was prepared by plotting the average of absorbent at 595 nm reading for each protein standards and its concentration mg/ml. A linear regression line was drawn passing though these points (Figure 12).

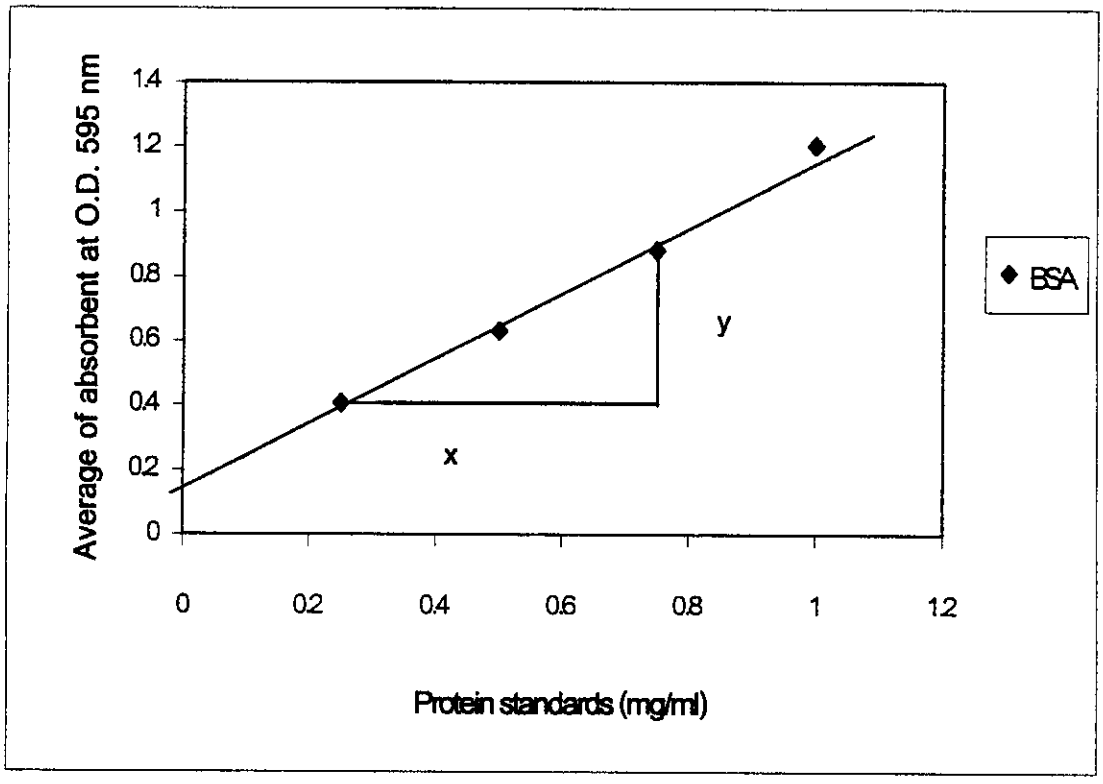


Figure 12 Standard curve for the Bio-Rad protein assay

Calculation the slope of regression from standard curve and determine the protein concentration of each unknown samples as below;

$$\frac{y}{x} = \text{Slope} = \frac{\text{Absorbence of standard}}{\text{Concentration of standdard}}$$

$$\text{Slope} = \frac{\text{Absorbence of unknown}}{\text{Concentration of unknown}}$$

$$\text{Concentration of unknown} = \frac{\text{Absorbence of unknown}}{\text{Slope}}$$

Appendix IV Method to determine the activity of enzymes

Standard curves of enzymes were prepared by plotting the average of absorbent form standard enzymes and product produced per min. A linear regression line was drawn and the slope of regression was calculated. Then, slope of standard enzyme was used to determine the product of enzyme of unknown samples, and to calculate the activity of enzyme as indicated below ;

$$\text{Product of enzyme} = \frac{\text{Absorbence of unknown}}{\text{Slope of enzyme standard}} \quad \text{(m-mole product/min)}$$

$$\text{Activity of enzyme} = \frac{\text{Product of enzyme}}{\text{1 mg protein}} \quad \text{(m-mole product/min/mg protein)}$$

Example from to reported for biochemical assays

No. of Sample	Protein			Alpha-esterase			Beta-esterase			MFOs		
	O.D.[1]	Concentration[2]	1mg PT[3]	O.D.[4]	Product of enzyme[5]	Activity[6]	4	5	6	4	5	6

Protein

1: O.D. = average of absorbence of sample

2: concentration = O.D. / slope of protein standard (mg/ml)

3: 1 mg PT = 1 / concentration

Alpha-esterase, Beta-esterase and MFOs

4: O.D. = average of absorbence of sample

5: product of enzyme = O.D. / slope of enzyme standard (m-mole product/min)

6: activity of enzyme = product of enzyme / 1mg PT (m-mole product/min/mg protein)