Monitoring and managing insecticide resistance in *Aedes* mosquito populations

Interim guidance for entomologists

World Health Organization

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Contents

Introduction

The use of safe and efficacious insecticides against the adult and larval populations of mosquito vectors is one of the most effective ways to rapidly interrupt transmission of Zika virus, as well as other viruses transmitted by *Aedes* mosquitoes such as chikungunya and dengue.

Insecticide resistance monitoring in field populations of *Aedes* is required to determine the levels, mechanisms and geographical distribution of resistance in order to select appropriate insecticides for vector control. Evidence-based decisions will ensure that effective insecticides are selected and used. Changes in insecticide susceptibility status should also direct policy and operational decisions.

Insecticide resistance monitoring is an essential part of entomological surveillance. Together with information on adult mosquito density, larval and pupal indices, ecology and habitats, and efficacy of vector control interventions, appropriate responses to prevent and control Zika virus and other mosquito-borne viruses can be developed.

This document summarizes WHO test procedures for the detection of insecticide resistance in *Aedes* larvae and adults including insect growth regulators (IGRs) and Bti products. It also outlines strategies to manage insecticide resistance in countries facing Zika virus and other viruses transmitted by this species of mosquito.

This document is for qualified entomologists at national and sub-national level who are responsible for evaluating the susceptibility status of local *Aedes* populations.

Procedures to test adult mosquitoes

Mosquito sampling and rearing

Representative sentinel sites need to be identified in Zika affected areas for the assessment of insecticide resistance status of *Aedes* populations. These should include neighbourhoods with the highest insecticide application in public health. Larval stages are easier to collect from the most productive breeding sites, and should be kept alive and taken to a local or centralized insectary facility for rearing. Usually the second or F2 generation is used, as enough number of larvae or adult mosquitoes are needed for the necessary tests.

Only female mosquitoes should be used in the tests. It is recommended that susceptibility tests be performed on non-blood fed females of 3–5 days old.

Conditions

The optimum conditions for phenotypic susceptibility tests are 27 ± 2°C temperature, 75 ± 10 % relative humidity and low illumination that are usually maintained in an insectary. Where such infrastructure is not available, the tests should be done indoors in a building free from insecticidal contamination while maintaining optimum humidity and temperature using local procedures and avoiding extreme illumination and wind. Where possible, subsequent comparison test should be made under similar conditions of temperature and humidity.

Materials

- 1 mosquito cage
- Live female mosquitoes (140 healthy specimens needed for testing)
- 14 plastic tubes (125mm L x 44mm D)
- 7 steel spring-wire clips
- 7 copper spring-wire clips
- Red marker, or 5 red dot stickers
- Yellow marker, or 2 yellow dot stickers
- 14 sheets clean white paper (12 x 15 cm)
- 5 sheets insecticide-impregnated paper
- 2 sheets oil-impregnated control paper
- 7 pads of cotton wool
- 10% sugar water solution
- 140 Eppendorf tubes

Procedures for susceptibility testing[1]

1. Prepare seven **holding tubes** by rolling seven sheets of clean white paper (12 x 15 cm) into a cylinder shape. Individually insert each cylinder into a separate holding tube, and fasten into position with a steel spring-wire clip. Attached the tubes to slides.
2. In each of the holding tubes, aspirate 20 active female mosquitoes (in batches) from a mosquito cage through the filling hole in the slide. After the mosquitoes have been transferred, close the slide unit and set the holding tubes in an upright position for one hour at optimum test conditions. After one hour, replace any knocked-down, dead or damaged mosquitoes with healthy ones.
3. Prepare seven additional tubes in the same manner as the holding tubes. Mark five with a red dot (**exposure tubes**) and two with a yellow dot (**control tubes**). Line each exposure tube with a sheet of insecticide-impregnated paper, and the two control tubes with a sheet of oil-impregnated control paper. Fasten the papers to each tube with a copper spring-wire clip.
4. Attach the five exposure tubes to the vacant position on the slides, and open the slide unit. Blow the mosquitoes gently into the exposure tubes. Once all the mosquitoes are in the exposure tubes, close the slide unit, detach the exposure tubes and set them upright. Fill the two control tubes with mosquitoes in the same way.
5. Keep the mosquitoes in the exposure and control tubes for one hour. Make sure that the tubes are set in an upright vertical position with the mesh-screen on top.
6. After one hour, transfer the mosquitoes back to the holding tubes by reversing the procedure outlined in Step 4. Set all the holding tubes upright, with the mesh-screen on top. Soak a pad of cotton-wool in 10% sugar water solution and place on the mesh-screen.
7. Maintain the mosquitoes in the holding tubes for a 24 hour recovery period, under the optimum conditions previously described. It is important to keep the holding tubes in a place free from extreme temperature and humidity, ideally in an insectary facility. Temperature and humidity should be recorded during the recovery period.
8. At the end of the 24 hour recovery period, count and record the number of dead mosquitoes (refer to Annex 1 for definitions of knockdown and mortality).

   If mosquito mortality in the control tubes exceeds 10%, correct the mortalities of all treated groups using Abbott's formula (below). Discard the test and repeat if the corrected mortality in the control tubes exceeds 10%.

$$\text{Corrected mortality (\%)} = \frac{\text{\% mortality with treated paper} - \text{\% mortality with control}}{100 - \text{\% mortality with control}} \times 100$$

9. If supplementary tests (biochemical or molecular) are necessary after completing the susceptibility test, transfer each mosquito (dead or alive) to an individual, clearly labelled Eppendorf tube. Refrigerate and store the tubes until they can be processed for supplementary testing.

Interpretation of results

In light of new knowledge and the need for prompt action to counter the spread of resistance among vector populations, guidance on interpreting the results of the WHO bioassay test has been revised. The current recommendations are as follows[1]:

- Mortality between 98–100%: Susceptibility is indicated
- Mortality less than 98%: Resistance suggested. Further tests are needed to verify.
- Mortality between 90%–97% (corrected if necessary): Presence of resistant genes in the vector population must be confirmed. The confirmation of resistance may be obtained by performing additional bioassay tests with the same insecticide on the same population or on the progeny of any surviving mosquitoes (reared under insectary conditions) and/ or by conducting molecular assays for known resistance mechanisms. If at least two additional tests consistently show mortality below 98%, then resistance is confirmed.
- Mortality less than 90%: Confirmation of existence of resistant genes in the test population with additional bioassays may not be necessary, as long as a minimum of 100 mosquitoes were tested. However, further investigation of the mechanisms and distribution of resistance should be undertaken.

Sourcing test kits and papers

Insecticide impregnated papers are currently prepared at University Sains Malaysia, Penang, Malaysia[1] (on behalf of WHO). The information on discriminating concentrations of insecticides is currently limited[2], therefore local tests may be carried out to establish baseline doses using test papers with serial doses that can be ordered with the facility in Penang, Malaysia.

As a routine, the papers are prepared only with the discriminating concentrations of the relevant insecticides and those are packed in plastic boxes; each box contains 8 papers. The equipment and/or insecticide impregnated papers can be ordered separately. Table 1 shows the discriminating doses for *Aedes*.

Given the logistical challenges of securing these supplies timely and the fact that these are needed to support the current Zika epidemic, exceptionally, other reputable sources – especially of test papers are encouraged to supply them.

Table 1. Discriminating concentrations and exposure time of insecticides used for *Aedes* mosquitoes[2]:

Insecticide class	Insecticide	Discriminating concentrations	Exposure period (hours)
Pyrethroids	Cyfluthrin	0.15%[b]	1
	Deltamethrin	0.03%[a]	1
	Lambdcyhalothrin	0.03%	1
	Permethrin	0.25%	1
	Etofenprox	0.5%[b]	1
	Alpha-cypermethrin	0.03%[a]	1
Organophosphate	Fenitrothion	1%	1
	Malathion	0.8%	1
	Pirimiphos methyl	0.21%[b]	1

[a] Tentative

[b] Determined for *Anopheles* mosquitoes[1], tentative for *Aedes*.

[1] Ordering instructions can be found at http://www.who.int/whopes/resistance/en/WHO_CDS_CPE_PVC_2001.2.pdf.

[2] For more information, see http://who.int/whopes/resistance/en/Diagnostic_concentrations_May_2014.pdf.

Procedures to test larvae

Larvae collection

Aedes larvae can be collected from the most productive breeding containers by using dippers or by the use of ovitraps. The larvae must be sorted into different instars in the insectary.

Conditions

The optimum conditions for larvae susceptibility tests are similar as for adult mosquitoes: a proper insectary facility in which enough larvae are reared for the tests is required. This should include a bench in which the testing cups can be maintained without disturbance during the tests. Note that for some insecticides such as insect growth regulators, the tests could last around 2 weeks. 27 ± 2°C temperature, 75 ± 10 % relative humidity and low illumination that are usually maintained in an insectary. Where such infrastructure is not available, the tests should be done indoors in a building free from insecticidal contamination while maintaining optimum humidity and temperature using local procedures and avoiding extreme illumination and wind. Where possible, subsequent comparison test should be made under similar conditions of temperature and humidity.

Materials

- 140 x 3th –4th instar larve
- 1 pipette capable of delivering 100–1000 µl
- 5 x 1 ml pipettes (insecticides)
- 1 x 1 ml pipette (control)
- 100 x 100 µl disposable tips
- 100 x 500 µl disposable tips
- 3 droppers with rubber suction bulbs
- 1 small strainer or a loop of plastic screen
- 7 disposable cups (if not available, use 120ml and 250ml glass bowls/beakers)
- 1 graduated measuring cylinder
- Data recording forms
- Log–probit software or paper
- Alcohol (or organic solvent)

Insecticide and bacterial larvicide susceptibility tests[3,4]

Mosquito larvae can be exposed to a wide range of insecticide concentrations to test the activity range of the insecticide. After determining the mortality of larvae in this range of concentrations, a narrower range (of 4–5 concentrations, yielding between 10% and 95% mortality in 24 or 48 hours) is used to determine LC50 and LC90 values[3]. The same procedure is carried out for a susceptible strain and a field test population.

Procedures for insecticide and bacterial larvicide testing

1. Dilute the appropriate volume of insecticide with 100ml or 200ml of water in disposable test cups or vessels to obtain the desired target dosage, starting with the lowest concentration.
2. Transfer 20 late 3rd/early 4th instar larvae to each cup. Small, unhealthy or damaged larvae should be removed and replaced.
3. After 24 hours of exposure, record larval mortality. For slow-acting insecticides, 48 hour exposure may be required. Count moribund larvae and add to numbers of dead larvae to calculate mortality percentage. 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 or not showing the characteristic diving reaction when the water is disturbed.
4. Four or more replicates are set up for each concentration and an equal number of controls are set up simultaneously with tap water, to which 1 ml alcohol (or the organic solvent used) is added. Each test should be run three times on different days.
5. If more than 10% of the control larvae pupate in the course of the experiment, the test should be discarded and repeated. If the control mortality is between 5% and 20%, the mortalities of treated groups should be corrected according to Abbott's formula (below).

$$\text{Corrected mortality (\%)} = \frac{\text{\% mortality in test} - \text{\% mortality in control}}{100 - \text{\% mortality in control}} \times 100$$

6. Lethal concentrations (LC) which result in 50%, 90% and 99.9% mortality (LC50, LC90 and LC99.9) values are calculated from a log dosage–probit mortality regression line using computer software programs, or estimated using log–probit paper.
7. When using a susceptible strain, twice the calculated LC99.9 is used as a discriminating or diagnostic concentration to test resistance in field populations. For example, the discriminating concentration for the insecticide temephos has been established as 0.012 mg/L. If a rapid assessment of the status of temephos resistance of a field population is required, this discriminating dose could be used.
8. Resistance ratios (RR) are often calculated and useful to monitor the evolution of insecticide resistance in a field population. The way to calculate a RR is to divide the LC50 of the field population by the LC50 of a susceptible strain. When RR is <5 the field population is considered susceptible, when RR is between 5 and 10 mosquitoes are considered to have moderate resistance, and when RR is >10 the mosquitoes are highly resistant.

[3] For details on how to prepare insecticide and bacterial larvicides solutions, refer to 'Guidelines for Laboratory and Field Testing of Mosquito Larvicides' [3].

Insect growth regulators (IGRs) tests[3]

IGRs have a delayed action on treated larvae. These juvenile hormone analogues interfere with the transformation of late instar larvae to pupae and then to adult, whereas chitin synthesis inhibitors inhibit cuticle formation and affect all instars and immature stages of the mosquito.

In IGRs tests, mortality is assessed every two or every three days until adult emergence, or mortality in immature stages. An accurate initial count of larvae is essential because of the cannibalistic or scavenging behaviour of larvae during the long exposure period. Larvae should also be provided with a small amount of food at two-day intervals until the test ends.

The effect of IGRs on mosquito larvae is expressed in terms of the percentage of larvae that do not develop into successfully emerging adults, or adult emergence inhibition (IE%). The same procedure is carried out for a susceptible mosquito strain and a wild/field population.

Procedures for IGR testing

1. Expose third instar larvae to a range of IGR concentrations, in the same way as chemical larvicide test procedures.
2. Count mortality or survival every two or every three days until the complete emergence of adults, or mortality in immatures.
3. At the end of the observation period, calculate the impact of the IGR (expressed as IE%) based on the number of larvae that do not develop successfully into viable adults. In recording IE% for each concentration, moribund and dead larvae and pupae, as well as adult mosquitoes not completely separated from the pupal case, are considered as "affected". The number of successfully emerged adults may also be counted from the empty pupal skins. The test is terminated when all the larvae or pupae in the controls have died or emerged into adults. Any deformities or morphogenetic effects that occur in moulting immature mosquitoes or emerging adults are also recorded.
4. Combine the data from all replicates of each concentration. Total or mean emergence inhibition can be calculated on the basis of the number of third stage larvae exposed. The overall emergence of adults reflects activity. IE% is calculated using the following formula:

$$IE\% = 100 - \frac{\%\text{ adults emerged in treated batches} \times 100}{\%\text{ adults emerged in control batches}}$$

5. If adult emergence in the control is less than 90%, the test should be discarded and repeated. Where the percentage is between 91% and 99%, correct the data using Abbott's formula.
6. Perform probit regression analysis on IE values obtained at each concentration to determine IE50 and IE90 values (using computer software programs or estimated from log–probit paper).
7. Resistance ratios (RR) can be used to monitor the evolution of insecticide resistance in a field population. Calculate a RR by dividing the IE50 of the field population by the IE50 of a susceptible strain. RR <5 indicates a susceptible field population; RR from 5—10 indicates moderate resistance; and RR >10 indicate high resistance[7].

Managing insecticide resistance

While non-insecticide based tools for controlling *Aedes* are available and should be prioritized, the use of insecticides is necessary as a rapid intervention to interrupt disease transmission, particularly during outbreaks of mosquito-borne disease. Few insecticide active ingredients are available for public health use, and to minimize the impact of insecticide resistance in a control programme, appropriate decisions need to be made. Usually, the first option to be selected by a control programme is the least expensive insecticide with highest effectiveness against the vector populations, which also has a low-risk to applicators and bystanders. Development of insecticide resistance drives a change to more expensive options, compromising coverage.

In the absence of insecticide pressure, insecticide resistance may be reduced or reverted. It is this reversion to susceptibility which is the underlying assumption behind any effective resistance management strategy. However, reversion rates are variable and may be very slow, particularly when a chemical insecticide has been used for many years.

Insecticide resistance management can be undertaken using insecticides in conjunction with other non-chemical insecticidal vector control methods. This approach is suitable for *Aedes* mosquitoes since different tools are available for adult and larval control, including chemical insecticides with unrelated classes/modes of action such as IGRs or biologically derived toxins (i.e. Bti, spinosad) for larval control.

Options to prevent or limit the development of insecticide resistance include[5,6]:

- **Insecticide rotation:** To preclude the emergence of resistance, insecticides of unrelated classes with different modes of action should be sprayed in rotation, ideally in a bi-annual cycle. This is good practice and should be implemented wherever possible, even before resistance has been reported.
- **Combining interventions:** As adult and larval control must be done simultaneously to have an impact on vector populations, different classes of insecticides with different modes of action should be used for each stage (e.g. using an organophosphate as an adulticide, combined with a Bti or Bti+Bs, or IGR as larvicides).
- **Mosaics:** One compound is used in one geographic area, and a compound of different insecticide classes and mode of action (i.e. organophosphate and pyrethroid) used in neighbouring areas. This strategy, while theoretically robust, is logistically difficult to implement – especially in an epidemic setting where rapid response is key to contain a disease outbreak.

Resistance management and mode of action

In order to successfully develop and implement rotation, combination or mosaic resistance management strategies, knowledge of the mode of action and/or chemical class of the available insecticide products is essential. Knowledge of the resistance mechanism developed by the local population of mosquitoes is also important, since cross-resistance may occur between different classes of insecticides that share the same resistance mechanism. Synergist assays, biochemical and molecular tests are available for this purpose. Collaborative work with academic or research institutes will help control programmes generate this useful information.

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Annex 1: Definition of knock-down and mortality for adult mosquitoes[4]

For the purpose of insecticide bioassays, the definition of knock-down[5] and mortality involves not only the state of the insect but also the time at which the observation is made.

A mosquito is classified as dead or knocked down if it is immobile or unable to stand or take off (Table 1). The distinction between knocked down and dead is defined only by the time of observation. The assessment of knock-down is made within 60 minutes after exposure. Mortality is determined at least 24 hours after exposure. The holding container may be tapped a few times before a final determination is made.

In the case of slow-acting insecticides, the recovery period may be extended beyond 24 hours. Control mortality should be measured over the same recovery period. Mortality after 24 hours should be recorded and, in some cases, repeated observations may be appropriate.

Table 2. Classification of adult mosquitoes in bioassays

Alive	Moribund*	Dead*
• Can stand on and fly in a coordinated manner	• Cannot stand (e.g. has 1 or 2 legs) • Cannot fly in a coord-inated manner • Lies on its back, moving legs and wings but unable to take off • Can stand and take off briefly, but falls down immediately	• No sign of life; immobile; cannot stand

* Knocked down after 60 minutes or dead after 24 hours of exposure

[4] Source : Report of the 15th WHOPES Working Group Meeting (http://apps.who.int/iris/bitstream/10665/75304/1/9789241504089_eng.pdf).

[5] Tthe criteria for knock-down and mortality are applicable to pyrethroids as well as other insecticides. For example, the criteria specified in the *Guidelines for laboratory and field testing of long-lasting insecticidal mosquito nets* (available at http://www.who.int/whopes/guidelines/en/) require minimum levels of knock-down and or mortality, not knock-down and mortality.