

Insecticide Resistance and CDC Bottle Bioassay Testing

1) What is insecticide resistance?

Insecticide resistance is a genetic selection to insecticides that allows some individual insects the ability to survive previously lethal doses of insecticides. Over time, this results in the survival of increasing numbers of resistant individual mosquitoes within the population and may impair the effectiveness of insecticide applications causing operational control failures.

2) Where does resistance occur?

Resistance generally occurs in areas where insecticide exposure is frequent and/or mosquitoes are exposed at increasing doses (long-term use of the same types of insecticides).

3) Does resistance occur throughout the area where mosquitoes are being controlled?

The level and mechanism(s) of resistance can be focal and often depend on many biological and operational factors, such as the flight range of the species and the frequency of applications. Do not assume that one location where resistant mosquitoes are found is representative of the larger region.

4) Can insecticide resistance be managed or reversed?

Mosquito populations can regain susceptibility to a particular insecticide if resistance is routinely monitored for and changes in mosquito control activities are made in a timely manner. The rate at which a population recovers is dependent on which genes are producing the resistance and their frequency the population.

5) What are some of the mechanisms of resistance?

Metabolic resistance results from changes in a mosquito's enzyme system that allows for a more rapid break-down and detoxification of the insecticide than normal, thus preventing the insecticide from reaching the target site. Enzymes include esterases, mono-oxygenases, and glutathione S-transferases.

Target site resistance occurs when receptors that the insecticide is designed to attack are altered by one or more mutations. The insecticide is no longer able to properly bind to the intended target site and thus the insect is unaffected or less affected by the insecticide. The two primary altered target sites include: 1) mutation(s) in the sodium channel receptor called knockdown resistance (*kdr*), which makes pyrethroid insecticides less effective, and 2) altered acetylcholinesterase (AChE), which makes organophosphate insecticides less effective.

Cross resistance can occur between different classes of insecticides that share the same mode of action.

6) What insecticide resistance tests are available?

CDC bottle bioassay test. This test is used to detect and characterize resistance to an active ingredient of an insecticide in a selected mosquito species. This test measures the time it takes for the insecticide to produce mosquito mortality. This bioassay indicates the proportion of the population that is resistant. The results are displayed (presented) as a time versus mortality curve. Resistance is assumed if there are any delays in mortality beyond the prescribed diagnostic time compared to the diagnostic dose. Inhibitors can be used to determine the mechanism behind resistance by exposing the mosquitoes to the inhibitors themselves for a set period of time followed by exposure to an active ingredient inside the bottles or by exposing the mosquitoes to the inhibitor and active ingredient in the same bottle. The assay can be statistically analyzed using a Weibel distribution curve or a Kaplan-Meier test.

Advantages: This assay is reliable and follows simple protocols that are inexpensive and effective. Bottle bioassays show resistance trends regardless of mechanism. Certain inhibitors can be used in conjunction with various active ingredients to determine the mechanisms at play, such as oxidase and the esterase groups.

Disadvantages: The bottle bioassay typically requires use of a known susceptible population (i.e., lab colony) of the same species for initial comparison to the field population. Although the use of a lab colony is only necessary for the initial assay or to calibrate bottles to determine what doses and times should be used for a particular compound. Once these are known, the comparison overtime is what should be used to make informed decisions depending on if resistance is increasing, decreasing, or staying the same over time. The use of inhibitors requires additional manipulation of the mosquitoes or the inhibitor may not have enough time to counteract the enzymes.

Dose-response bioassay test. This assay can also be set up in glass bottles and is used to determine the level of resistance to an active ingredient of an insecticide in a specific mosquito species. Bottles or individual mosquitoes are treated with a series of doses/concentrations of the active ingredient, and mortality is assessed as an endpoint of a specific time period (usually 24 hours). The results are visualized by logistic regression or probit statistical test. This assay is used widely in toxicology and pharmacology. In vector control it is most commonly used to test immature mosquito stages to larvicides, but can be used for testing adults and adulticides.

Advantages: Assay results can be statistically analyzed (using probit analysis or logistic regression) and level of resistance (resistance ratio) can be quantified. Synergists can be used to determine different mechanisms of resistance, but mosquitoes must be pretreated.

Disadvantages: Easy to follow visual protocols are not currently available. There must be a susceptible colony available to calculate resistance ratio. Finding the appropriate series of doses requires additional time and mosquitoes. Greatest confidence in results is at the 50% lethal level. For control purposes >95% mortality is the goal.

Kdr (knockdown resistance). Knockdown resistance is a mechanism of resistance caused by mutations in the mosquito's DNA, which results in reduced sensitivity of the nervous system to the effects of pyrethroid insecticides. *Kdr* testing is complementary to other resistance tests available.

Advantages: *Kdr* testing provides more detail about the genetic "profile" of a wild population showing resistance to pyrethroids than can be obtained through field trials or use of the CDC bottle bioassay. The test results provide precise information on the frequency of the resistance gene when mosquitoes are tested individually.

Disadvantages: Presence of *kdr* in a mosquito population does not necessarily equate to control failures in the field. *Kdr* is a recessive gene and therefore heterozygous individuals (those with one copy of the mutation and one copy of the susceptible gene) do not express this resistance. The gene can be detected with the assay but in some cases operational control may not be affected by the detection of *kdr* in a population. To determine the "impact" of *kdr* on controlling a given mosquito population, further investigation is recommended (such as investigations using CDC bottle bioassays and field cage trials). The currently available protocol for California species can test for *kdr* resistance in *Culex pipiens* and *Cx. quinquefasciatus* mosquitoes.

Biochemical Assay or Microplate Assay. This test evaluates metabolic resistance mechanisms to a variety of insecticides by measuring levels of particular enzyme types in mosquitoes. In addition, an insensitive acetylcholine esterase can be performed using this format. This particular assay detects the ACE1 mutation that confers OP resistance. Results can be read visually in the lab without the use of specialized equipment to determine whether resistance is absent or present with most of the assays. The Glutathione-S-transferase must be read using a plate reader. By using a microplate reader for all enzymes, more precise results can be obtained. Microplate assays are complementary to the other resistance tests available.

Advantages: Multiple assays can be run on the same mosquito sample. Cross resistance within individual and populations of mosquitoes can be examined. The esterase, oxidase, and insensitive acetylcholine esterase tests are rapid and accurate.

Disadvantages: To quantify the data, expensive equipment, such as a spectrometer, is required. Mosquitoes must be freshly killed immediately before an assay or stored frozen at -70 to preserve enzymes until the assay is run.

These tests provide valuable insight into how the different insecticides will behave in a given mosquito population. Knowing the mechanisms is important in determining how to alter integrated vector management (IVM) strategies and predict how mosquitoes will react to the various types of insecticides.

CDC BOTTLE BIOASSAY TEST

7) What information does the CDC bottle bioassay test provide and how is it useful?

Results from CDC bottle bioassays can be used to detect resistance at a given time and location within a specific mosquito species to a particular active ingredient or formulated insecticide. Ideally, this is done by initially comparing field-caught or reared mosquitoes to a known susceptible population or previous tests from the same location.

Consistently monitoring for resistance helps determine changes in susceptibility of wild populations over time. The data from resistance tests can be used to make resistance management decisions before an insecticide is no longer useful.

8) How many mosquito populations or areas should be tested?

Since resistance can be localized, it is best to monitor a variety of sites or habitats, particularly where disease transmission occurs and/or where high insecticide use is known or suspected.

9) How can I obtain a susceptible mosquito population?

Susceptible *Culex pipens* and *Cx. tarsalis* colonies are maintained at some vector control agencies or university facilities. Contact the California Department of Public Health for more information.

10) Can you test wild caught mosquitoes if you do not have a susceptible colony of the same species for comparison?

Yes, bottle tests that are done consistently on the same population from the same geographic location over time will produce data that show increases or decreases in resistance levels with a population. Resistance is not static and is reversible if it is detected early and managed.

11) What are the reference parameters?

Diagnostic dose and diagnostic time are unique for each insecticide and each mosquito species. Once they have been determined, they should be used for every subsequent test for that population. These parameters must remain steady to detect changes in susceptibility over time.

a) What is the diagnostic dose?

The diagnostic dose is the lowest concentration of insecticide with 100% mortality or knockdown of the susceptible mosquitoes in a given time (typically between 30 and 60 minutes).

b) What is the diagnostic time?

It is the expected time for the diagnostic dose of the insecticide to achieve 100% mortality or knockdown of susceptible mosquitoes, which confirms susceptibility. For example, if susceptible colony A mosquitoes were all dead at 30 minutes post-exposure to a diagnostic dose of 21.5 µg/bottle, then 30 minutes would be the diagnostic time used for comparison with wild caught mosquitoes. Individuals still “flying” in the bottle after this time are considered to have resistance to the insecticide tested.

12) Why does the bottle bioassay use different diagnostic doses for different active ingredients?

The rate at which an insecticide works is dependent on the intrinsic toxicity of the molecule, how well it penetrates the mosquito, and other factors.

13) Can you use formulated insecticides in the bottle bioassay instead of technical grade active ingredient to test for resistance?

Formulated product bioassays also use diagnostic doses to indicate resistance. Bottles need to be calibrated for diagnostic time and dose for each product regardless of the active ingredient in order to run a CDC bottle bioassay. However, formulated products contain inert and other ingredients, which can change the mosquitoes' reaction to an active ingredient and may mask resistance. Technical grade chemicals are routinely used in California to test for resistance to active ingredients found in common insecticides. This method is preferred since there are several years of comparable data available to interpret changes in resistance patterns in California. The CDC protocols reduce the need to calibrate bottles by working with technical grade chemicals.

INTERPRETING CDC BOTTLE BIOASSAY TEST RESULTS

14) How is a bottle bioassay test conducted?

Bottles are treated with a known concentration (i.e., the diagnostic dose) of an insecticide. The insecticide coats the inner wall of the bottle. Mosquitoes from field populations and susceptible colony mosquitoes are placed in the treated bottles. At specific time intervals the numbers of dead mosquitoes are counted in each bottle. These data are used to plot time-mortality data on a graph and compare the susceptible colony to the field population. More importantly, the data can be compared to previous data to look for trends over years.

15) What is the time-mortality data and what does it mean?

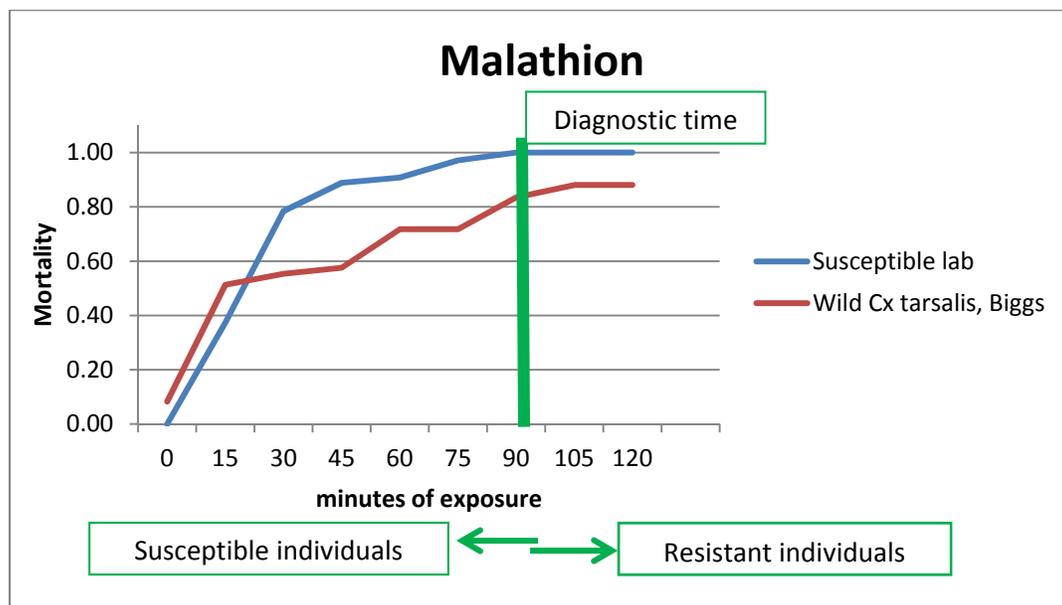
These data measure the mortality of the mosquito populations at specific points in time when the mosquitoes are treated with a diagnostic dose of insecticide. If some of the wild caught mosquitoes survive longer than the threshold time it is indicative of resistance. Additionally, if comparing a wild caught population to itself over time, changes in the shape of the time-mortality curve document further selection or recovery of that population.

16) How do I interpret the data graphs?

The diagnostic time is measured by drawing a straight line down from the point where 100% of the susceptible mosquitoes died (see graph below). The mosquitoes that die before the diagnostic time are considered to be susceptible. Mosquitoes that survive beyond the diagnostic time represent the proportion of resistant individuals in the population. In the example below, 12% of the individual mosquitoes tested are possibly resistant to malathion.

17) How do I interpret the resistance rate?

Generally, a 10% resistance proportion in the vector population is deemed acceptable, and in such situations the active ingredient used can still be an effective control method. The World Health Organization recommends the following interpretations of mortality at the prescribed diagnostic dose and diagnostic time: 100-98% mortality = susceptible; 98-90% mortality = possibly resistant (more testing required); and <90% mortality = confirmed resistant (more testing required). An observed mortality rate below 98% indicates the presence of resistant genes in the vector population. Confirmation may be obtained by performing additional CDC bottle bioassays and conducting molecular and biochemical assays for known resistance mechanisms. Two additional tests that yield similar results can confirm that the population is resistant.



OPERATIONAL STRATEGIES

18) Does the CDC bottle bioassay correlate with operational control applications?

Bottle bioassay tests cannot mimic operational control applications because the diagnostic doses used in bottle bioassays are not comparable to label rates used in the field. Field tests (cage trials) can be used to simulate operational control applications and may indicate the presence of resistance if both wild caught and susceptible mosquitoes are used. Bottle bioassays complement field tests. Together they give a more complete picture of how mosquitoes respond to a particular active ingredient or formulated insecticide product.

19) Does evidence of resistance from a bottle bioassay test predict application failures in the field?

Bottle bioassay results for an active ingredient do not necessarily correlate with or predict efficacy of a formulated product applied in the field. Bottle bioassays are designed to detect the presence of resistance in mosquito populations before efficacy of commercial products are compromised. The detection of resistance and the mechanisms associated with resistance do not indicate that the particular product or active ingredient are no longer useful, but may indicate the potential for decreased mosquito control efficacy. Control efficacy will eventually diminish as the level of resistance increases in local mosquito populations.

20) If bottle bioassay results indicate resistance, should we make other operational changes?

Low levels of resistance may not affect operational control but should be regularly monitored. The best way to manage resistance is to remain flexible with operational strategies and use IVM. This can be accomplished by adulticiding less frequently and increasing larval control. Another option is to rotate classes of chemicals but be aware of potential cross resistance. Other strategies include mixing chemicals, spraying a higher concentration of the active ingredient mixed with a synergist, using chemicals with less residual action, controlling focally resistant populations with biological control agents, or changing treatment thresholds. Failure to adopt IVM strategies to combat resistance may lead to increased resistance over time and control failures in the future.

21) How often should mosquitoes be tested for resistance?

Ideally, surveillance for insecticide resistance should be conducted at the beginning and end of each mosquito season. At a minimum, resistance surveillance should be conducted yearly on select populations.

22) Why should we continue to do resistance testing?

The goal is to detect stability or changes in insecticide susceptibility over time. To accurately describe resistance in a population, regular testing is critical. Routine testing is the only method to detect changes in a population in a timely manner so that changes in operational actions can be made before commercial products fail.

For more information, including submission of mosquitoes for *kdr* testing, contact the California Department of Public Health, Vector-Borne Disease Section, at 916-552-9730.

Publications

Brogdon, W.G., and Chan, A., Guide for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle Bioassay. Web 24 October 2014.

http://www.cdc.gov/malaria/resources/pdf/fsp/ir_manual/ir_cdc_bioassay_en.pdf

WHO. Test Procedures for Insecticide Resistance Monitoring in Malaria Vector Mosquitoes. Geneva, World Health Organization, Web 14 November 2013.

<http://www.who.int/malaria/publications/atoz/9789241505154/en/>

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