CONUS Manual for Evaluating Insecticide Resistance in Mosquitoes Using the CDC Bottle Bioassay Kit

U.S. Department of Health and Human Services
Centers for Disease Control and Prevention

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Contents

Intended Audience

Vector control professionals

Objectives

The primary objective is to provide guidance for using the CDC bottle bioassay as a surveillance tool for detecting insecticide resistance in mosquito vector populations. This document is intended for state and local public health officials and vector control specialists.

PREFACE

Insecticide resistance in a vector population is initially detected and characterized by using some sort of bioassay such as the World Health Organization larval or adult bioassay, topical assays, field cage tests, or the CDC bottle bioassay to determine whether a particular insecticide is effective against local mosquito species. Ideally, this essential question should be answered before a specific insecticide formulation is chosen and procured for vector control.

The Centers for Disease Control and Prevention (CDC) bottle bioassay is a surveillance tool for detecting insecticide resistance in vector populations. The assay is designed to help determine if the active ingredient in an insecticide formulation may be effective against a local mosquito population at a specific location at a given time. This information, combined with results of bioassays using enzyme inhibitors and those of biochemical and molecular assays, can assist in determining the resistant mechanism(s) present and guide decisions of which insecticide to use.

The aim of this document is to provide a practical laboratory manual that describes how to perform and interpret the CDC bottle bioassay for CONUS. Information for resistance testing can also be obtained from the CDC website at https://www.cdc.gov/zika/vector/insecticide-resistance.html.

We hope you find this tool useful in the support of vector control programs.

Sincerely,

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GUIDELINES

1. Introduction

Insecticide resistance is defined as a genetic change in response to selection by toxicants that may impair control in the field (Sawicki, 1987). Insecticide resistance is believed to develop largely, if not entirely, because of the natural selection of pre-adaptive variants that possess genetically controlled mechanisms for detoxification, target-site insensitivity, or other means of survival in the presence of an insecticide. Resistance develops at different rates between species and even between populations of the same species due to genetic, reproductive, biological/ecological, and operational factors.

Only two classes of insecticides, pyrethroids and organophosphates, are available for adult mosquito control in the continental United States. Reports of insecticide resistance within the United States have been submitted to the Arthropod Pesticide Resistance Database (APRD) maintained by Michigan State University (http://www.pesticideresistance.org/) for *Culex quinquefasciatus* (82), *Cx. pipiens* (20), *Cx. tarsalis* (14), and *Aedes aegypti* (4). These figures are not a true representation of the current resistance status of mosquito populations within the United States, as routine monitoring does not normally occur across the nation. This manual will describe the Centers for Disease Control and Prevention (CDC) bottle bioassay, a tool for detecting resistance to insecticides. The information provided by this bioassay, combined with results of bioassays using enzyme inhibitors and those of biochemical and molecular assays, can also assist in determining mechanism(s) associated with resistance.

The CDC bottle bioassay relies on time-mortality data, which measures the time it takes an insecticide to penetrate a vector, traverse its intervening tissues, get to the target site, and act on that site. Anything that prevents or delays the compound from achieving its objective—killing insects—contributes to resistance (**Figure 1**). Information derived from the CDC bottle bioassay may provide initial evidence that an insecticide is losing its effectiveness. This methodology should be considered for routine use even before an insecticide is procured for vector control.

The CDC bottle bioassay can be performed on vector populations collected from the field or on those reared in an insectary from larval or egg collections.

The CDC bottle bioassay is simple, rapid, and economical compared to other alternatives. It can also be used as part of a broader insecticide resistance monitoring program, which may include field cage tests and biochemical and molecular methods.

The CDC bottle bioassay can be used for any mosquito species.

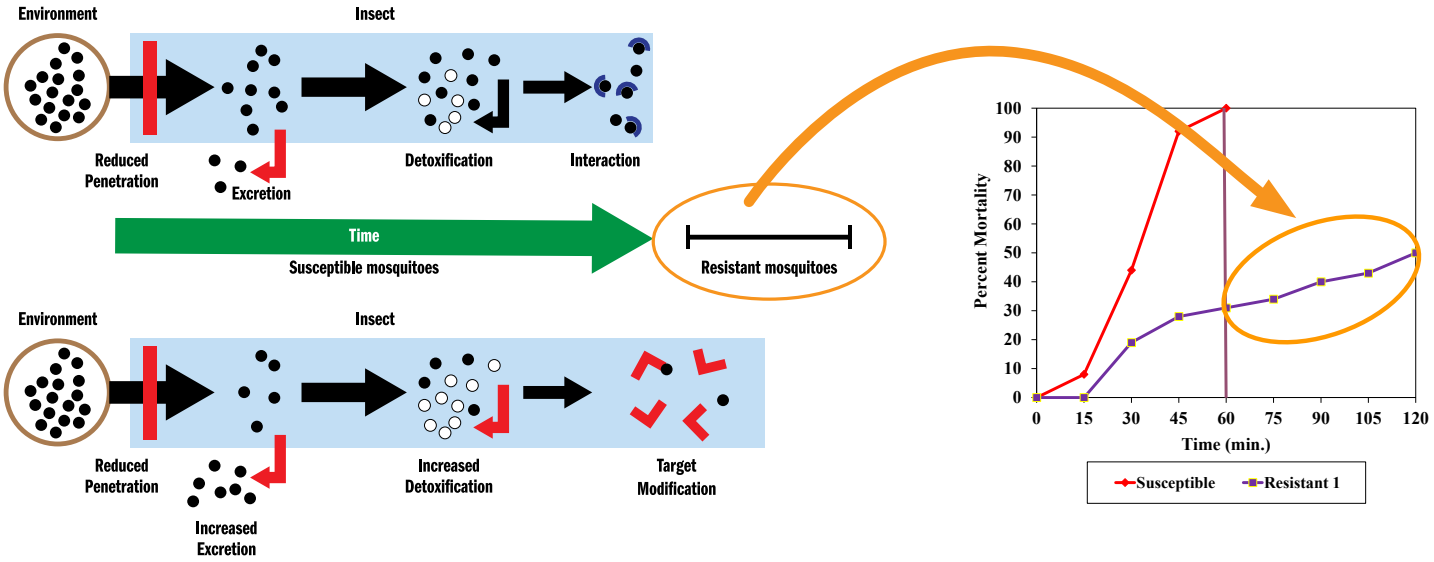


Figure 1. Insecticide pathways in susceptible (top) and resistant mosquitoes (bottom). The CDC bottle bioassay detects differences in mortality at the diagnostic time between susceptible and resistant mosquitoes. (Yellow circles highlight threshold time, beyond which mosquitoes are exhibiting resistance). Black dots represent insecticide molecules.

2. Materials and reagents

2.1. Materials provided in CDC bottle bioassay kit (Figure 2)

- 15, 250-ml Wheaton bottles with PTFE lined lids;
- Graduated disposable plastic pipettes;
- Standard mouth aspirator;
- Clear 50 ml conical tube for measuring acetone or absolute ethanol;
- Amber 50 ml conical tube(s) for insecticide solutions;
- Conical tube holder;
- Parafilm®;
- Blue lab mat;
- CDC bottle bioassay protocol;
- Data sheet example (electronic data sheet will be emailed);
- Safety Data Sheets (SDS) for requested insecticides.

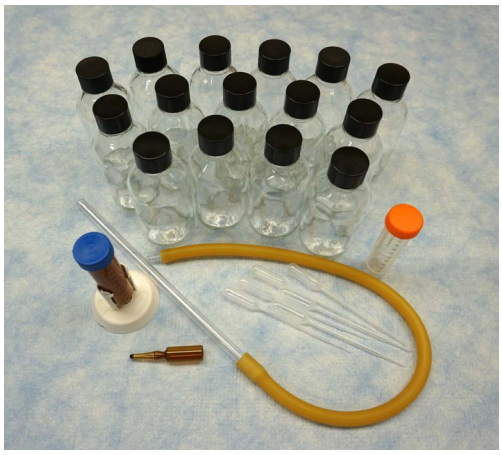


Figure 2: Materials and reagents included in the CDC bottle bioassay kit.

2.2. Materials provided by user

- Containers for transferring/holding mosquitoes;
- Timer capable of counting seconds;
- Permanent markers for labeling bottles, caps, and pipettes;
- Masking tape or colored label tape for labeling bottles, caps, and pipettes;
- Disposable gloves;
- Data sheets, pens, and pencils for data recording;
- Safety goggles (see note below);
- Lab coat (see note below).

2.3. Reagents provided in CDC bottle bioassay kit

- Requested technical grade insecticides and enzyme inhibitors.

2.4. Reagents provided by user

- Acetone or technical grade absolute ethanol.

2.3. Biological material provided by user

- Adult mosquitoes for testing.

Note: Use safety procedures as recommended by your institution when handling insecticides (e.g., gloves, laboratory coat, and safety goggles).

3. Initial considerations

3.1. Mosquito handling

Mosquitoes to be used in the bioassay can be collected as adults from the field (of mixed age and physiological status) or as adults of a known age reared from field larval or egg collections. If field-collected adults are used, their physiological status (i.e., unfed, blood fed semi-gravid, gravid) should be recorded on the data sheet. Both female and male mosquitoes may be used in the bioassay. It is recommended that a minimum of 100 mosquitoes, divided among four replicate bottles, should be tested for an insecticide. When it is not possible to collect this number of mosquitoes on a single occasion, results of multiple bioassays over a few days may be pooled to achieve the recommended sample size, 100 mosquitoes. In either case, each bioassay must include a control bottle with 10–25 mosquitoes in addition to the insecticide bottles.

Some field collections may contain different species. In those situations where different mosquito species exist, it is recommended that species be identified, either before or after the CDC bottle bioassay. If a predominant species is detected (i.e., more than 95% belong to one single species), consider this the species tested, and the results of the CDC bottle bioassay can be considered adequate for the predominant species.

3.2. Diagnostic dose and diagnostic time

The diagnostic dose is a dose of insecticide that kills 100% of susceptible mosquitoes within a given time. The expected time for the insecticide to achieve this objective is called the diagnostic time. Those are the reference points against which all field mosquito results are compared. Resistance is assumed to be present if a significant portion of the test population survives the diagnostic dose at the diagnostic time.

At the CDC in Fort Collins, CO, the diagnostic doses and diagnostic times have been determined for the susceptible *Aedes aegypti* REX, *Ae. albopictus* LC, *Cx. pipiens* New York, *Cx. quinquefasciatus* Seabring, and *Cx. tarsalis* KNWR mosquito colonies **(Table 1)**. The diagnostic doses and the diagnostic times in Table 1 serve as sample reference points for the main active ingredients used in the continental United States.

Table 1. Sample diagnostic doses and diagnostic times for technical grade insecticides

Insecticide	Insecticide concentration (µg/bottle)	*Ae. aegypti*	*Ae. albopictus*	*Cx. pipiens*	*Cx. quinquefasciatus*	*Cx. tarsalis*
		Diagnostic time per species (minutes)	Diagnostic time per species (minutes)	Diagnostic time per species (minutes)	Diagnostic time per species (minutes)	Diagnostic time per species (minutes)
Chlorpyrifos	20	45	45	90	45	60
Deltamethrin	0.75	30	30	45	60	--
Etofenprox	12.5	15	30	15	30	60
Fenthion	800	--	--	75	45	45
Malathion	400	15	30	45	45	45
Naled	2.25	30	30	45	45	45
Permethrin	43	10	10	30	30	30
Prallethrin	0.05	--	--	60	60	--
Pyrethrum	15	15	30	45	45	30
Sumethrin	20	10	45	30	45	30

Note: If piperonyl butoxide (PBO), S.S.S-tributylphosphorotrithioate (DEF), or diethyl maleate (DM) were requested, these are to be used as inhibitors to determine the presence of metabolic resistance mechanism(s). See Section 5.

3.3. Preparation of insecticide solutions

The bottles used for the bioassay are coated inside with the diagnostic dose of the insecticide under evaluation. As can be seen in **Table 1,** the diagnostic dose is a predetermined amount of insecticide per bottle.

1. To make insecticide solutions to coat bottles, using the clear 50 ml conical tube in the CDC bottle kit, measure out 50 ml of acetone or absolute ethanol;
2. Transfer acetone/alcohol to an amber conical tube;
3. Using a disposable pipette, transfer 1 ml of acetone/ethanol from amber conical tube to amber vial containing technical grade insecticide. Mix several times by pipetting up and down and then transfer mixture inside vial back to amber conical tube. Repeat 3 times;
4. Put the lid on the amber conical tube and mix solution in it by turning it upside down 5-8 times. It is important to label the amber conical tube containing the insecticide solution with the name of the insecticide and date of preparation. Once the insecticide solution is made, it can be stored in the refrigerator (4°C) for future use;
5. Seal the tube with Parafilm before storing by wrapping the Parafilm around the lid and the area of the tube directly beneath the lid.

It is recommended to take the insecticide solutions out of the refrigerator at least 1 hour before running the bioassay to allow them to come to room temperature before use. The insecticide solution should be turned upside down 5-8 times before use to mix it.

3.4. Marking of bottles

1. Since the bottles will be reused, consider using a piece of masking tape on the bottles and caps for marking them instead of writing directly on the bottles and caps **(Figure 3**). This may facilitate the cleaning of the bottles after the bioassay is completed;
2. Mark one bottle and its cap as the control;
3. Mark the other four bottles and caps with the replicate number (1–4);
4. If more than one type of insecticide or more than one concentration of the insecticide is being tested at the same time, also label the bottles and their caps with the insecticide name;
5. Mark both the cap and the bottle so that bottles are associated with their respective caps. This is important to ensure each bottle is treated with the same amount of insecticide.

3.5. Bottle coating

1. Make sure that bottles and caps are completely dry;
2. Remove caps from the bottles;
3. Label one disposable pipette as “acetone/ethanol” for the control bottle, and another pipette with the insecticide name being used to coat the test bottles;
4. Add 1 ml of acetone/ethanol to the control bottle and put the cap back on tightly;

Figure 3. Labeling bottles and caps.

5. Add 1 ml of insecticide solution to the first test bottle **(Figure 4).** Put the cap back on tightly;
6. Repeat Step 5 with the other three test bottles;
7. Swirl the contents inside the first test bottle so that the bottom is coated **(Figure 5)**;
8. Turn the bottle upside down and swirl to coat the inside of the cap **(Figure 6)**. Repeat Steps 7–8 for the other thee test bottles;
9. Place the bottle on its side for a moment to let the contents pool. Gently roll the bottle so that the sides all the way around are coated **(Figure 7)**;
10. Repeat this for all bottles;
11. Remove the caps and continue rolling bottles on their side until all visible signs of the liquid are gone from inside, and the bottles look completely dry **(Figure 8)**;
12. Leave uncapped bottles on their sides and cover both the bottles and caps with something that will keep them protected from light. Examples of materials could be blue lab mat, cardboard box, towel, etc.;
13. If bottles are not used right away, store bottles in a dark place (such as a drawer) with the caps off. More information on the storage of coated bottles is given in **Section 4.3.**

3.6. Procedures for cleaning and drying bottles between uses

1. Wash the bottles with warm soapy water and rinse thoroughly with water at least 3 times. Soaps that do not produce many suds are easier to rinse thoroughly. Tap water can be used for this step;
2. Leave bottles to dry completely at room temperature or in the sun, with the caps off. In humid situations, bottles can be left to dry with caps off overnight or longer;
3. If you want to ensure that the cleaning procedure is adequate, introduce some susceptible mosquitoes into a sample of recently washed and dried bottles. Mosquitoes should not die right away. If they do, repeat the washing and drying procedure.

Figure 4. Adding insecticide solution to test bottle.

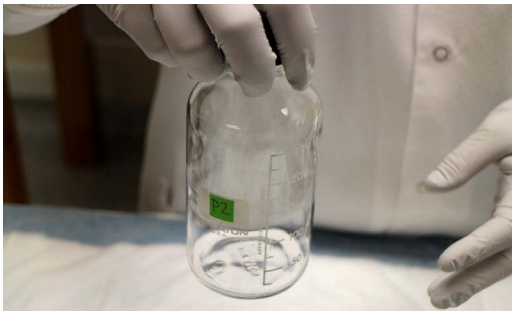


Figure 5. Coating the bottom of the bottle.

Figure 6. Coating the cap of the bottle.

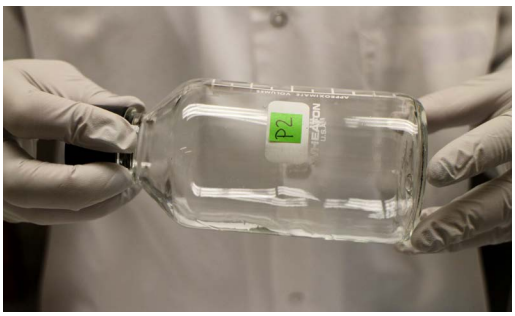


Figure 7. Coating the sides of the bottle.

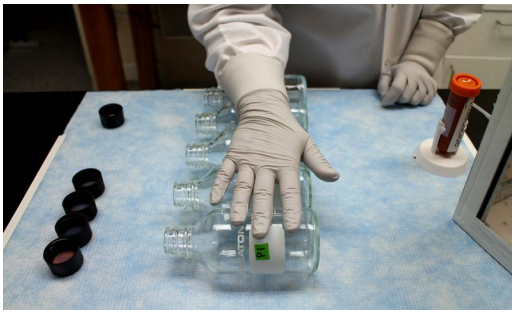


Figure 8. Rolling bottles to evaporate liquid.

4. CDC bottle bioassay method

4.1. Bioassay procedure

The bioassay can be performed with the bottles in an upright position or with the bottles lying on their sides. The important thing is to be consistent and follow the same procedure each time.

The steps:

1. Place lids gently on tops of bottles. Using an aspirator, introduce ~25 mosquitoes into the control bottle by gently blowing to expel the mosquitoes **(Figure 9**). Immediately screw the cap on the bottle. **Note:** Blowing too hard can damage or kill the mosquitoes in the transfer process. The number of mosquitoes does not have to be exact;
2. Introduce ~25 mosquitoes into each test bottle; again, the exact number does not matter;
3. Start a timer for 5 minutes. Be sure to examine the bottles at Time 0 and count the number of mosquitoes that may have died during the transfer process; if you find dead mosquitoes at Time 0, make a note of them on the data sheet;
4. Record how many mosquitoes are dead or alive, whichever is easier to count, at 5 minutes, 10 minutes, then every 15 minutes until all are dead, or up to 2 hours. It is not necessary to continue the bioassay beyond 2 hours; mosquitoes are considered dead if they can no longer stand. See **Box 1** for more information;
5. Input data into Excel data sheet (attached in the email);
6. The Excel bottle data spreadsheet will graph the total percent mortality (Y axis) against time (X axis) using a linear scale;
7. Remember that mortality at the diagnostic time is just one critical value because it represents the threshold between susceptibility and resistance. It is important to run the assay for the full 2 hours as long as there are live mosquitoes present. Refer to Table 1 for diagnostic doses and times for commonly used insecticides;
8. Take into consideration mortality in the control bottle at the end of the bioassay when reporting the results of the bioassay (Section 4.5). Use Abbott's formula to correct results if the mortality at 2 hours in the control bottle is between 3% and 10%. You may need to discard the bioassay results if mortality in the control bottle at the end of the test was >10%. An Excel bottle data spreadsheet that will automatically correct the results using Abbott's formula is available upon request from USBottleAssayKit@cdc.gov.

Figure 9. Aspirating mosquitoes into a bottle.

BOX 1

Notes about mortality criteria

- "Dead" mosquitoes are mosquitoes that cannot stand, have erratic behavior, or fly up and then fall down. This indicates that the insecticide has reached its target site. Mosquitoes do not have to be stone cold dead.
- It helps to gently rotate the bottle while taking the count. Tap on the bottle to see if mosquitoes can't right themselves or have difficulty flying.
- Immobile mosquitoes that slide along the curvature of the bottle can be easily categorized as dead.
- It is easier to count mosquitoes by holding the bottle above eye level and looking up through the bottle.
- It is easier to count the number of dead mosquitoes in the first readings of the bioassay, and it is easier to count the number of live mosquitoes when few remain alive.
- In the end, the percentage of dead mosquitoes at the diagnostic time (dead mosquitoes/total of mosquitoes in the assay) is the most important value in the graph.

4.2. General considerations

1. Be careful not to touch the inside of the bottle with the aspirator, as this may contaminate the aspirator;
2. Remember that the number of mosquitoes in the each of the test bottles does not need to be equal. You will calculate a percentage of mortality across all bottles.
3. Start the timer when the first or last bottle receives its mosquitoes. It is important to be consistent and follow the same timer start procedure each time.
4. Mosquitoes alive at the diagnostic time **(Table 1)** represent mosquitoes resistant to the insecticide being tested.
5. The shape of the curve beyond the diagnostic time is also important. Make note of when 100% mortality is reached or if it is not reached within 2 hours, what final percent mortality is achieved.

4.3. Handling of coated bottles

More than one batch of mosquitoes can be run in a single bottle in one day. However, the main limiting factor for reusing previously coated bottles is moisture build-up with successive introductions of mosquitoes, especially in humid conditions. If the bottles are to be reused on the same day, it is necessary to leave some time (1–2 hours, longer if in a humid climate) between the bioassays for the bottles to dry out (with caps off) before introducing more mosquitoes. If the bottles are to be reused the following day, bottles with caps off can be left to dry overnight protected from direct light.

If the bottles are not to be used soon after coating them with insecticide, it is recommended to let them dry with their caps off. When the bottles are dry, they should be stored in a dark place (such as a drawer) with their caps removed. Depending on the insecticide used, bottles can be stored from 12 hours to 4 days in this manner. The length of time bottles can be stored depends on the insecticide. Naled and Pyrethrum break down quickly with oxygen, so they should be used immediately after being prepared. Organophosphate-coated bottles should be used within 2–4 days. Pyrethroid-coated bottles should be used within 1–3 days. To check if a stored bottle is still acceptable, it is possible to put some mosquitoes known to be susceptible in the bottle. If they die in the expected time frame (within the diagnostic time), the bottle can still be used. Bottles can be coated in a central laboratory and shipped for use in the field. During transport, bottles should have their caps on.

4.4. Identification of mechanisms of resistance

Resistance is assumed to be present if a portion of the test population survives the diagnostic dose at the diagnostic time (Figure 1). Additional mosquitoes from the same test population can be used to identify the mechanism(s) of resistance present using modifications of the CDC bottle bioassay **(Section 5),** enzymatic assays, or molecular methods. Mosquitoes should be stored at -80°C if enzymatic testing is to be conducted.

4.5. Validity of the data

With practice, the mortality of mosquitoes in the control bottle at the end of the bioassay should be zero. In most cases, mortality of up to 3% in the control bottle may be ignored. In cases where mortality is 3%–10% in the control bottle, it is possible to either use Abbott's formula to correct the findings (see **Box 2**), or discard results and repeat the bioassay. If mortality in the control bottle is greater than 10% at the end of the bioassay, the results of this particular run should be discarded, and the CDC bottle bioassay should be repeated. If a particular mosquito collection is essentially irreplaceable and the bioassay cannot be repeated, Abbott's formula can be considered even when control mortality is >10%.

BOX 2

Abbott's formula

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

For example: If mortality in test bottles is 50% at diagnostic time and control mortality is 10% at 2 hours, the corrected mortality is [(50%–10%)/(100%–10%)] × 100 = 44.4%

Note: In cases of 100% mortality in test bottles, Abbott's formula has no effect. For example:

[(100% − 10%) / (100% − 10%)] × 100 = 100% corrected mortality

5. Variations of CDC bottle bioassay to determine resistance mechanisms

5.1. Background

The CDC bottle bioassay using bottles coated with a single active ingredient provides information on insecticide resistance to that particular insecticide in adult vectors. This data may provide early evidence that an insecticide is losing its effectiveness.

A rapid and inexpensive follow-up, once resistance is detected, is to assess which resistance mechanisms are involved by using the CDC bottle bioassay with enzyme inhibitors. Enzyme inhibitors are available for the metabolic detoxification enzymes: esterases, oxidases, and glutathione s-transferases.

Inhibitors act by abolishing the apparent resistance observed in the CDC bottle bioassay if a detoxification enzyme plays a role in that particular resistance mechanism (**Figures 10a and 10b**). Data for resistant and susceptible populations are shown (**Figure 10a**). Once an inhibitor is used on the resistant population, one of three things might happen (**Figure 10b**):

a. Resistance to the insecticide is abolished (time-mortality line A), which suggests that the mechanism related to that inhibitor is playing a role in the insecticide resistance observed;

b. Resistance to the insecticide is partially abolished (time-mortality line B). This suggests that the mechanism related to that inhibitor is involved in the resistance, but it is not the only mechanism involved in this particular case;

c. Resistance to the insecticide is unaffected (time-mortality line C). This indicates that the mechanism related to that inhibitor is not involved in the resistance.

5.2. Use of inhibitors

Common inhibitors used in conjunction with the CDC bottle bioassay:

- Piperonyl butoxide (PBO), which inhibits oxidase activity;
- S.S.S-tributlyphosphorotrithioate (DEF), which inhibits esterase activity;
- Diethyl maleate (DEM), which inhibit glutathione transferase activity.

Testing mosquitoes with inhibitors is a two-step procedure. Mosquitoes are first exposed to the inhibitors(s) for 1 hour and then held for 1 hour. Mosquitoes are then tested to determine the presence of metabolic mechanism(s) using the CDC bottle bioassay.

Fig. 10a.

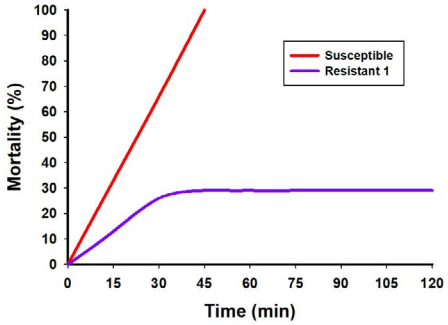
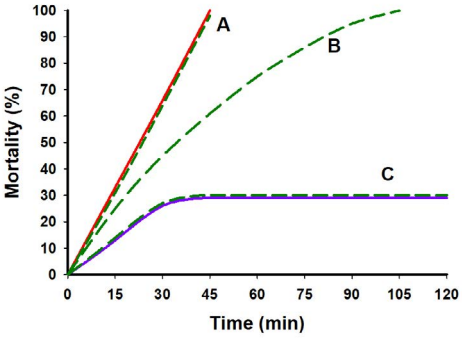


Fig. 10b.



Figures 10a and 10b. Effects of inhibitors on resistant vector populations. **Figure 10a** shows data for a population of resistant vectors compared to a susceptible population. **Figure 10b** shows the three possible outcomes of inhibitor exposure (**Line A:** Resistance to the insecticide is abolished; **Line B:** Resistance to the insecticide is partially abolished; and **Line C:** Resistance to the insecticide is unaffected).

5.3. Preparation of bottles for inhibitor bioassays

To use the bioassay with inhibitors (see **Table 2**):

1. To make the inhibitor solutions, use the clear 50 ml conical tube in the CDC bottle kit to measure out 50 ml of acetone or absolute ethanol;
2. Transfer to amber conical tube;
3. Using a disposable pipette, transfer 1 ml of acetone/ethanol to amber vial containing technical grade inhibitor. Mix several times by pipetting up and down and transfer to amber conical tube. Repeat Step 3, 3 times;
4. Mix solution in amber conical tube by turning it upside down 5–8 times. It is important to label the amber conical tube containing the inhibitor solution with the name of the inhibitor and date of preparation;
5. Once the inhibitor solution is made, it can be stored in the refrigerator (4°C) for future use. Seal the tube with Parafilm before storing;
6. Mark one bottle and its cap as the inhibitor-exposure bottle;
7. Add 1 ml of inhibitor solution to the inhibitor-exposure bottle and put the cap back on tightly;
8. Coat the bottles, remove the caps, and let the bottles dry as in **Section 3.6**;
9. Prepare a set of bottles with insecticide to which resistance was found in mosquito population **(Section 3.6).**

5.4. CDC bottle bioassay with inhibitor

To run the CDC bottle bioassay with inhibitor:

1. Introduce ~125 mosquitoes into the inhibitor-exposure bottle;
2. Keep the mosquitoes in the bottles for 1 hour to allow the inhibitor to act;
3. After the 1-hour exposure is completed, transfer the mosquitoes to a holding container;
4. Hold mosquitoes for 1 hour before running CDC bottle bioassay with insecticide;
5. Perform the CDC bottle bioassay as in **Section 4** using a set of insecticide-coated bottles (one control and 4 test bottles) using the inhibitor-exposed mosquitoes;
6. Compare the data from the inhibitor-exposed mosquitoes to the original insecticide-exposed mosquitoes. An Excel spreadsheet that will graph inhibitor-exposed mosquito data is available upon request from USBottleAssayKit@cdc.gov.

5.5. Interpretation of bioassays with inhibitors

Section 6.1, and **Figures 10a** and **10b** provide information on how to interpret the results of the CDC bottle bioassay using inhibitors. Resistance that cannot be attributed to one of the detoxification mechanisms after all inhibitors have been used is likely to be due to a target site mechanism, such as knockdown resistance (kdr) or insensitive acetylcholinesterase.

Table 2. Inhibitor concentrations used in the CDC bottle bioassay

Inhibitor	Inhibitor concentration (µg/bottle)
Diethyl maleate (DM)	80
Piperonyl butoxide (PBO)	400
S.S.S-tributlyphosphorotrithioate (DEF)	125

5.6 Expression of knockdown resistance (kdr)

It is also possible to determine if a target site mechanism, such as the presence of the kdr gene (sodium channel mutation) is involved. It is crucial in areas where pyrethroids are used to evaluate the relative role of detoxification and target site mechanisms involved in a particular incidence of resistance. Knockdown resistance (kdr) confers pyrethroid cross-resistance, while a detoxification mechanism may or may not indicate cross-resistance. Knowledge of the resistance mechanism(s) involved is necessary to select a replacement insecticide formulation.

5.5 Procedure for assessing kdr expression

To determine the presence of knockdown resistance (kdr), transfer mosquitoes exposed to pyrethroids in the bioassay to a separate holding container, provide sugar water, and hold for 24 hours. Transfer the mosquitoes when all are dead or at the end of the test (2 hours) if some are still alive. Mosquitoes can be knocked down with CO_2 or by chilling them if they are still alive at the end of the assay. Record the number of mosquitoes that are alive at 24 hours. If the number of alive mosquitoes increased after 24 hours, this indicates that the kdr mechanism is being phenotypically expressed.

6. General interpretation of results

As with other resistance bioassays, data from the CDC bottle bioassay using test mosquitoes needs to be compared with data from susceptible mosquitoes or from a population that will serve as a baseline. Alternatively, if no baseline or susceptible data exists for a particular species, you can compare previous data generated from the same site over time or within a geographic area to spot trends both temporally and geographically. Calibration entails determining the diagnostic dose and the diagnostic time for a particular species in a given region, which correspond to the dose and time at which all of susceptible mosquitoes die (**Figure 11**). If test mosquitoes survive beyond this threshold, these survivors represent a proportion of the population that has something allowing them to delay the insecticide from reaching the target site and acting. In other words, they have some degree of resistance. In the example shown in **Figure 11,** all mosquitoes that died before the diagnostic time (light purple line) when exposed to insecticide-coated bottles were susceptible. Test mosquitoes surviving beyond the diagnostic time threshold are assumed to have some degree of resistance. In the example, only 30% of the test population was susceptible.

Recommendations for interpretation of bioassay data are shown in **Box 3.** The most important information is the mortality at the diagnostic time, but the bioassay should be carried out beyond the diagnostic time to evaluate the strength of resistance in the population. If 100% mortality is achieved at some point beyond the resistance threshold, as in the resistant 2 population (blue line), then the strength of the mechanism is not as great as if 100% mortality is never achieved, as in the resistant 1 population (dark purple line). It is important to note that

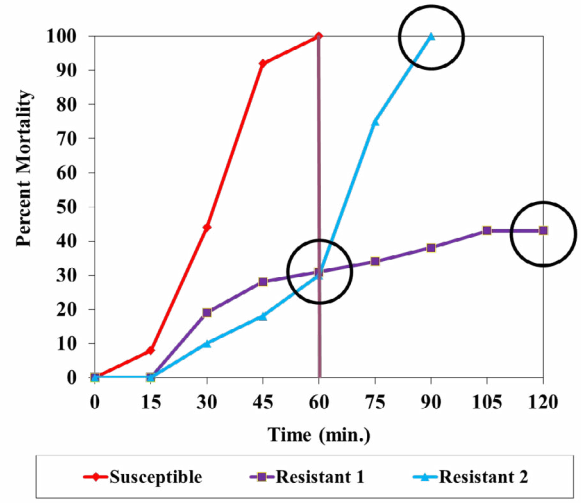


Figure 11. Example of two resistant populations (dark purple and blue lines) compared to a susceptible population (red line). Circles draw attention to thresholds of importance.

BOX 3
Interpretation of data for management purposes

World Health Organization (WHO) recommendations for assessing the significance of detected resistance:

- 97%–100% mortality at the recommended diagnostic time indicates susceptibility;
- 90%–96% mortality at the recommended diagnostic time indicates the population is developing resistance.
- <90% mortality at the recommended diagnostic time implies resistance.

resistance detected with this test does not necessarily translate into control failure. This test is much more sensitive in detecting the development of resistance than relying on failure in the field. The CDC bottle bioassay allows you to determine if resistance is present in a population before you lose the use of a chemical. Management decisions can be made in a timely enough manner to preserve susceptibility to that chemical.

7. Resistance surveillance

7.1. Background

Although resistance data are often collected as part of vector control programs, this is not done as regularly as it should be in a true resistance surveillance effort. Surveillance requires the regular collection and interpretation of data to support changes in control programs. It is important to consider the CDC bottle bioassay an instrument to collect information to support an insecticide resistance surveillance system. Resistance data are most valuable when collected over time to allow for comparisons and for monitoring of trends. It is important to consider how information collected as part of an insecticide resistance surveillance system will be used.

7.2. Features of resistance emergence

Several genetic, biologic, and operational factors influence the development of insecticide resistance. In many respects, resistance is a complex problem, with different outcomes possible in a particular area, depending on the influence of diverse factors on initial conditions. Even so, certain factors affect resistance development throughout the United States. Major resistance characteristics are discussed below, showing why each manifestation of resistance is potentially unique and therefore must be evaluated on case-by-case basis.

7.3. Focal nature of resistance

Vector control personnel frequently assume that resistance in a particular species occurs throughout their control area, but insecticide resistance can be focal. Generally, areas of ongoing vector control activities tend to have higher levels of resistance; when resistance levels in adjacent areas are compared, levels may be higher in areas of more intensive mosquito control. Alternatively, other uses of insecticides may also have an impact on the development of resistance. Areas with high levels of urban pest control can also have pockets of resistance in the absence of routine mosquito control.

7.4. Resistance and disease control

In some cases, vector control strategies in a given area may not be affected by the level of insecticide resistance. For example, a control program may be able to control only 75% of the vector population. In these cases, an insecticide resistance level lower than 10% will likely not affect disease control efforts. In such a situation, it would be sufficient to

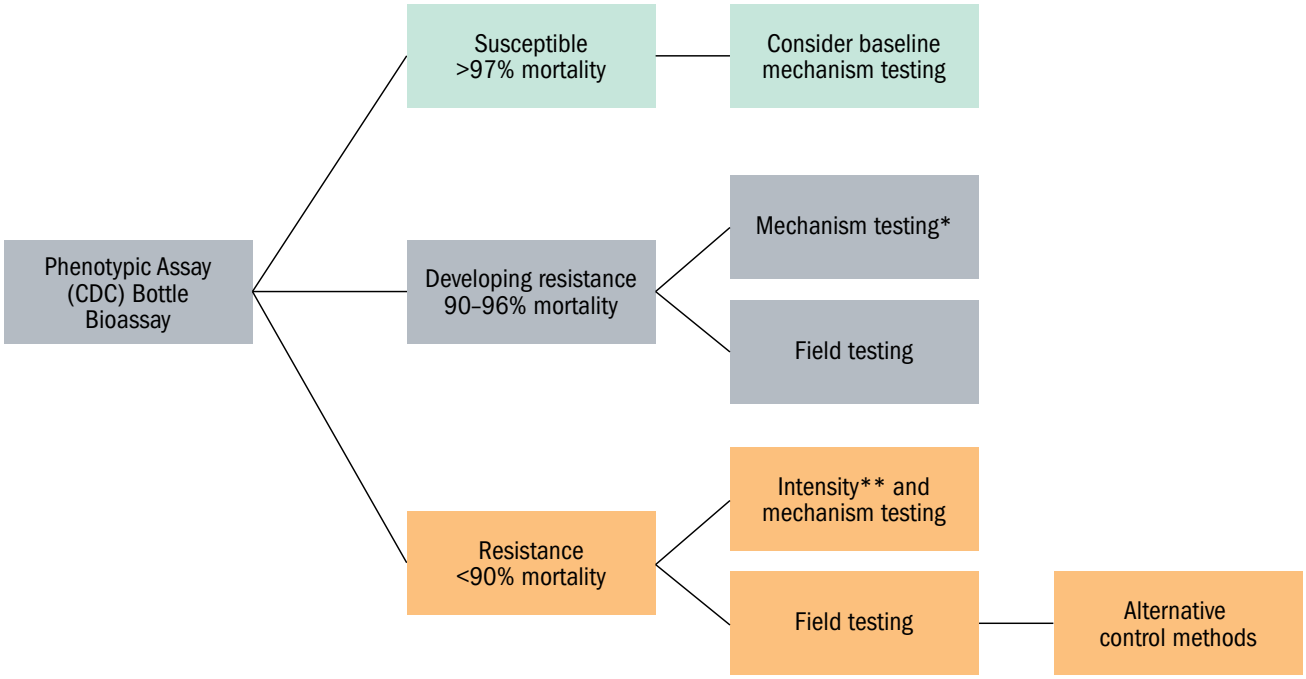
increase surveillance and monitor the level and frequency of resistance but no change in control strategies would be needed.

7.5. Guiding principles

In general terms, resistance surveillance should be conducted in areas where disease transmission is a concern and where insecticide-based control measures are contemplated, ideally before purchase of an insecticide formulation. In addition to constraints imposed by economic resources, the number of sites that can be sampled is highly dependent on the size of the area contemplated for insecticide use. Due to the potential focal nature of resistance, efforts must be made to choose spatially distributed sites in the area of interest, if possible. For example, with a species that does not have a large flight range such as *Cx. pipiens* or *Cx. quinquefasciatus,* areas 1.25 miles or more apart should not be assumed to have similar resistance patterns. Another means of deciding on surveillance sites is to focus on those areas of active virus transmission. Even if only one or a few sites can be monitored, this is far preferable to having no surveillance sites. In addition, efforts should be made to evaluate sites routinely, since comparative data is the most meaningful information.

Ideally, each site should be monitored once a year. Where control efforts are seasonal, it may be useful to monitor at the beginning and at the end of the control season. If several vectors in the area are seasonal, the resistance testing schedule should be adjusted to the species of interest.

Once resistance is detected with the CDC bottle bioassay, try to identify the resistance mechanism by using the CDC bottle bioassay with inhibitors or with biochemical and/or molecular methods (**Figure 12**). Deciding which insecticide to use will depend upon the specific mechanism(s) of resistance.



* Mechanism testing options: enzymes, molecular assays, CDC bottle bioassay with inhibitors.

** Intensity testing (strength of the resistance mechanism) can be done by looking at mortality at 120 minutes or by running bottles with 1X, 2X, 5X, and 10X the diagnostic dosage of insecticide.

Figure 12. Suggested algorithm for further testing depending on level of resistance detected in the CDC bottle bioassay.

8. Bibliography

1. Brogdon, WG and McAllister JC, 1998. Simplification of adult mosquito bioassays through use of time-mortality determinations in glass bottles. J. Am. Mosq. Control Assoc. 14(2):159–64.
2. National Center for Infectious Diseases, Centers for Disease Control and Prevention (CDC). Evaluating mosquitoes for insecticide resistance: Web-based instruction. Available from: http://www.cdc.gov/malaria.
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APPENDIX

Appendix 1. Frequently asked questions (FAQs)

1. What happens if there are not enough mosquitoes for a complete bioassay?

When the number of mosquitoes captured in the field is insufficient for a full bioassay (1 control and 4 coated test bottle), you can reduce the number of bottles to be tested, but each bioassay must ALWAYS be run with a control until the required number is completed. If the testing takes place over a long period of time, use recently coated bottles if necessary. See expected lifetime of coated bottles in the guideline (p. 14). Except in the case of naled and pyrethrin-coated bottles, coated bottles can be used multiple times over several days until the bioassay is completed, as long as moisture build-up from aspiration does not become excessive.

2. Should some bottles be designated solely as control bottles?

No, some bottles should not be designated as control bottles. Bottles should randomly be assigned as test or control bottles. This will provide an additional quality control to the adequacy of the washing procedure.

3. What if there are no susceptible mosquitoes available for CDC bottle bioassay calibration?

The diagnostic dose and diagnostic time for a particular species in a given area are similar. Use the diagnostic dose and the diagnostic time published in this guideline or consult the authors of this guideline or other users with experience in the method for that particular vector. Note that the value of the CDC bottle bioassay lies in showing changes over time in the characteristics of vector populations. Therefore, a baseline is useful even if some individual mosquitoes show resistance when the initial baseline is established.

When working with species that cannot be colonized and for which no susceptible information is available, one can use threshold times for more closely related species when looking at baseline data. As more information is gathered in subsequent assays, changes over time will become apparent and a better threshold time can be determined.

4. How can mosquitoes be introduced into the bottle without letting other mosquitoes escape?

In our experience, a swift decisive puff of air and screwing the cap on immediately will introduce mosquitoes without loss. Attempting to introduce mosquitoes into a bottle more than once will allow some of those already in the bottle to escape. This sometimes happens if the user attempts to put exactly the same number of mosquitoes into each bottle, which is not necessary.

5. What happens if there are fed and unfed mosquitoes among the field-collected mosquitoes to be used in the bioassay?

A collection of mosquitoes from the field may contain female mosquitoes in various physiological states, e.g., fed and unfed mosquitoes. There are three ways to deal with this. First, mosquitoes can be separated into fed and unfed cages. Second, they can be randomly selected. Alternatively, mosquitoes can be held for 1 or 2 days for the blood meal to be digested and then used for the bioassay. Mosquitoes that have fed and are digesting blood and making eggs will look resistant in the assay. This is because enzyme classes produced for blood digestion are the same as those that produced in insecticide resistance. The segment of a population that are digesting blood and developing eggs are also resting in protected areas. This segment is not targeted by ULV spraying. ULV spraying targets mosquitoes that are flying at the time of application.

6. Why do you also include males in the assay, shouldn't only females be tested?

Both females and males contribute genes equally to their progeny. It has been found that the resistant phenotype in males leads to higher rates of reproductive success, cryptic female selection of resistance genes, and improved capacity in sperm competition when compared to susceptible males.

7. Can I use formulated products instead of technical grade in bottle bioassays?

CDC does not recommend the use of formulated products as they can mask the development of insecticide resistance. However, there are several protocols available that have calculated the amount of formulated product that can be used in the bottle bioassay, but the amount is based on the active ingredient only. These amounts are often too great leading to 100% mortality in less than 5 minutes. Most of these formulations have to be recalibrated to achieve a diagnostic dose that gives a diagnostic time around 45–60 minutes. For example, Biomist® 4+4 ULV consists of 4% permethrin, 4% piperonyl butoxide (PBO), and 92% other ingredients. Published protocols calculated the diagnostic dose as 43 µg based on the permethrin alone. This amount resulted in 100% mortality at 10 minutes. It was determined in the lab upon recalibrating Biomist® 4+4 ULV, than an appropriate diagnostic dose is 0.5 µg with a diagnostic time of 60 minutes. This is an 86-fold decrease. Formulated products are often synergized with PBO and contain inert ingredients that are not listed on the label. Both synergists and inert ingredients contribute to the effectiveness of the product and can be insecticidal. PBO inhibits non-specific esterase and P450s and can mask resistance if this metabolic mechanism is playing a role in resistance. To determine whether a formulated product is effective at controlling a particular mosquito population, field cage tests should be conducted.

8. Can I add PBO from the test kit to the pyrethroids I ordered to mimic formulated product?

No, when ordering the CDC bottle kit, it is not appropriate to order PBO and add it with insecticides. The PBO on the order sheet is calibrated to determine if Cytochrome P-450 oxidases are the mechanism involved in resistance detected by the CDC bottle bioassay. PBO along with the other inhibitors should be ordered as a group to determine which metabolic resistance mechanism is being expressed in resistant population.

9. What's the next step if resistance is found in a population?

Depending on the level of resistance in the bottle bioassay, further testing to determine the resistance mechanism(s) present should be conducted (Figure 12). If an institution doesn't have the capacity to do additional testing, several state health departments or larger mosquito districts have the capabilities. The authors of this protocol from the CDC in Fort Collins, CO, can also be contacted to provide further assistance or advice.

10. What if a diagnostic dose and diagnostic time is not listed in Table 1 for the species I'm interested in testing?

If a susceptible colony is available for the species of interest, the bottle bioassay can be run using the listed diagnostic doses to determine a diagnostic time. If a susceptible colony isn't available, an initial bottle bioassay can be run using the species. Data collected over time can be compared to the initial bottle bioassay data to see if any changes are taking place in the species that may or may not warrant changes in control measures.

11. How do I dispose of chemical solutions?

Institutions have different ways of handling chemical waste. Contact your institution's safety officer to determine appropriate disposal methods. Mosquito control districts can add solutions to ULV tank and use for control purposes.