Guideline for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle Bioassay

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PREFACE

Insecticide resistance in a vector population is initially detected and characterized by using some sort of bioassay to determine whether a particular insecticide is able to control a vector at a given time. Ideally, this fundamental question should be answered before a particular insecticide is chosen and procured for vector control.

The Centers for Disease Control and Prevention (CDC) bottle bioassay is a surveillance tool for detecting resistance to insecticides in vector populations. It is designed to help determine if a particular formulation of an insecticide is able to control a vector at a specific location at a given time. This information, combined with results of bioassays using synergists and those of biochemical and molecular assays, can assist in determining which insecticide should be used if resistance is detected.

The aim of this document is to provide a practical laboratory guideline that describes how to perform and interpret the CDC bottle bioassay. Information for resistance testing can also be obtained from the CDC website at http://www.cdc.gov/malaria.

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

Sincerely,

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GUIDELINE

1. Introduction

Bioassays allow for the detection and characterization of insecticide resistance in a vector population. This guideline 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 synergists and those of biochemical and molecular assays, can also assist in determining mechanisms associated with resistance.

The CDC bottle bioassay relies on time mortality data, which are measures of 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. 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 considered, and 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 field collections. It is not recommended to use mosquitoes that have emerged from eggs laid in the insectary.

A major advantage of this bioassay is that different concentrations of an insecticide may be evaluated. Furthermore, the technique is simple, rapid, and economical compared to other alternatives. The CDC bottle bioassay can be used as part of a broader insecticide resistance monitoring program, which may include the World Health Organization (WHO) paper-based bioassay, and biochemical and molecular methods.

The CDC bottle bioassay can be used for any insect species. For the purposes of this guideline, mosquitoes will be used as an example.

2. Material and reagents

2.1. Material

- 250-ml Wheaton bottles with screw lids (Figure 1). Each bioassay typically requires five bottles: four for replicates and one for control;
- Graduated disposable plastic pipettes that can measure 1 ml, or micropipetters and tips;

- Aspirator apparatus for collecting mosquitoes;
- Containers for transferring/holding mosquitoes;
- Bottles for stock solutions. These can be amber-colored or foil-wrapped if clear bottles are used (100–1,000 ml depending on the user's choice of stock solution volume);
- Timer capable of counting seconds;
- Permanent markers for labeling bottles, caps, and pipettes;
- Masking tape for labeling bottles, caps, and pipettes;
- Disposable gloves;
- Sheets, pens, and pencils for data recording.

2.2. Reagents

- Insecticide(s) to be tested (technical grade or formulations);
- Acetone or technical grade absolute ethanol.

2.3. Biological material

- Mosquitoes for testing.

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

Figure 1: Material and reagents for the CDC bottle bioassay.

3. Initial considerations

3.1. Diagnostic dose and diagnostic time

The first step in standardizing the CDC bottle bioassay is to determine the diagnostic dose and the 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 other 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.

The diagnostic dose and the diagnostic time should be defined for each insecticide, each region, and each vector species that is monitored. The diagnostic dose and the diagnostic time are validated using a susceptible population of vectors collected from the field. Once the diagnostic dose and the diagnostic time for a species from a given location have been determined, these parameters should be used for testing that particular vector population from that location from that time on. Use of the same parameters is required to detect changes in the response of the population over time (e.g., number of test mosquitoes surviving after an exposure time that originally killed 100% of the test population). Detailed information about diagnostic doses, diagnostic times, and calibration of the CDC bottle bioassay is given in Appendix 2.

Diagnostic doses and diagnostic times have been determined for mosquitoes from many geographical regions. Table 1 shows diagnostic doses and diagnostic times applicable to *Anopheles* and *Aedes* mosquito populations. The diagnostic doses and the diagnostic times for anophelines shown below were agreed upon for use on anophelines collected in South America as part of the Amazon Malaria Initiative (AMI). These doses and times, as well as those listed for *Aedes*, are well within the range of diagnostic doses and diagnostic times for use worldwide. Therefore, the diagnostic doses and the diagnostic times in Table 1 serve as sample reference points for the main insecticides used globally. Diagnostic doses and diagnostic times for other insect species may still need to be determined. In

Table 1: Sample diagnostic doses and diagnostic times for *Anopheles* and *Aedes* mosquitoes.

Insecticide	Insecticide concentration per species (µg/bottle)		Diagnostic time (minutes)
	Anopheles	***Aedes***	
Bendiocarb	12.5	12.5	30
Cyfluthrin	12.5	10	30
Cypermethrin	12.5	10	30
DDT	100	75	45
Deltamethrin	12.5	10	30
Fenitrothion	50	50	30
Lambdcyhalothrin	12.5	10	30
Malathion	50	50	30
Permethrin	21.5	15	30
Pirimiphos-methyl	20	—	30

summary, determining the diagnostic dose and the diagnostic time is the first step to standardize the CDC bottle bioassay. This step should be done at the national or regional level in a given country or region to allow for comparability among different laboratories over time. Once the diagnostic dose and diagnostic time are agreed upon for a particular insecticide and mosquito species, there is no need to redo this exercise until evidence of high levels of resistance in this species is documented.

3.2. Preparation of stock solutions

The bottles used for the bioassay need to be coated inside with the diagnostic dose of the insecticide under evaluation. As can be seen from Table 1, the diagnostic dose is a determined amount of insecticide per bottle. Therefore, if 12.5 µg of deltamethrin is to be added to a test bottle, it would be advisable to have a stock solution with 12.5 µg/ml, which means that 1 ml of the solution would contain the desired amount of insecticide to be added to the bottle. This is equivalent to saying that it is practical to make stock solutions with concentrations that can be easily correlated to the dose needed to coat the bottles.

To make insecticide stock solutions, dilute the appropriate amount of insecticide (technical grade or formulation) in acetone or technical grade ethanol. Examples of quantities of technical grade insecticide needed to prepare 100 ml, 500 ml, and 1,000 ml of stock solutions are shown in Table 2. Technical grade insecticide may be solid or liquid and need to be of good quality and not be expired. It is important to label the stock solution bottle with the name of the insecticide, concentration, and date of preparation. Examples of preparation of stock solutions from technical grade and formulations are shown in Box 1. Once the stock solution is made, it can be stored in the refrigerator (4°C) in light-proof bottles (amber-colored bottles or foil-wrapped if clear) for future use. At the CDC, refrigerated stock solutions of many insecticides have been used for 2–3 years without degradation of activity. It is recommended to take the stock solutions out of the refrigerator at least 1 hour before running the bioassay to allow them to come to room temperature before use. The stock solution should be gently swirled before use to mix it.

Table 2: Quantities of technical grade insecticide required for preparation of different volumes of stock solution.

Insecticide	Weight (mg) of technical grade insecticide needed per volume of stock solution (*Anopheles*)			Weight (mg) of technical grade insecticide needed per volume of stock solution (*Aedes*)		
	100 ml	500 ml	1000 ml	100 ml	500 ml	1000 ml
Bendiocarb	1.25	6.25	12.5	1.25	6.25	12.5
Cyfluthrin	1.25	6.25	12.5	1	5	10
Cypermethrin	1.25	6.25	12.5	1	5	10
DDT	10	50	100	7.5	37.5	75
Deltamethrin	1.25	6.25	12.5	1	5	10
Fenitrothion	5	25	50	5	25	50
Lambdcyhalothrin	1.25	6.25	12.5	1	5	10
Malathion	5	25	50	5	25	50
Permethrin	2.15	10.75	21.5	1.5	7.5	15
Pirimiphos-methyl	2	10	20	—	—	—

Box 1: Examples of stock solution preparation.

1. Preparing stock solutions from technical grade insecticide

Assume a 100% deltamethrin technical grade insecticide. To obtain a concentration of 12.5 µg/bottle, dissolve 12.5 mg the insecticide in enough acetone or absolute ethanol to make 1 liter of total solution. Each 1 ml of this solution will contain 12.5 µg of the insecticide. Stock solutions of varying volumes (for example 100 ml) can be made for the convenience of the user as long as the proportion of insecticide and solvent remains constant.

2. Preparing stock solutions from concentrations other than technical grade

To calculate the volume of a formulation to be added to the solvent to reach the desired concentration in the stock solution, it is necessary to consider the concentration of the active ingredient in the formulation. To do that, simply divide the desired amount of milligrams needed for 1 liter of the stock solution by the concentration in the formulation available. This will give the volume of the formulation needed to make 1 liter of stock solution. The formula:

$$\frac{\text{Milligrams of technical grade}}{\text{\% of active ingredient in formulation}}$$

Example:

Using a formulation of 10% deltamethrin, calculate how much is needed to obtain 12.5 mg.

$$\text{Volume needed} = \frac{12.5\ \text{mg}}{0.10} = 125\ \text{mg of the 10\% formulation}$$

So, 125 mg of the 10% concentration of deltamethrin will need to be added to enough acetone or absolute ethanol to make 1 liter of total solution. By doing so, each 1 ml of the stock solution will contain 12.5 µg of deltamethrin.

3.3. Mosquito handling

Female 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 collections. Use of mosquitoes that have emerged from eggs laid in the insectary is not recommended. If field-collected adults are used, their physiological status (i.e., unfed, blood fed semi-gravid, gravid) should be recorded on the result sheet. Female mosquitoes should be fed only with 10% sugared solution the day before testing. It is recommended that a minimum of 100 mosquitoes, divided among four replicate bottles, should be tested for an insecticide at a given concentration. 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.

Some field collections may contain different species. Therefore, species must be identified either before or after the bioassay is conducted to validate its results (Box 2). To determine the species composition of mosquito collections before the bioassay, it is possible to "knock down" (anesthetize) mosquitoes with ethyl acetate.

Box 2: Guidelines for situations where different mosquito species exist in sample collections.

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.

If no particular species represents at least 95% of the mosquitoes being tested, account for this heterogeneous population. To achieve this:

1. Identify the species and sort before the bioassay using ethyl acetate. Conduct separate bioassays for each, or predominant, species; or
2. Start the bioassay without pre-identification if this is not possible (lack of expertise with mosquito "knock down," or presence of closely related or cryptic species). If there are surviving mosquitoes at the diagnostic time, stop the bioassay and separate live from dead mosquitoes. Identify the species for both live and dead mosquitoes, and consider them separately for analysis.

3.4. Procedures for cleaning and drying bottles before coating

a) Wash the bottles with warm soapy water and rinse thoroughly with water at least three times. Tap water can be used for this step;

b) Place bottles in an oven (50°C) for 15–20 min or until they are thoroughly dry before using them;

c) If there is no oven, 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;

d) To assure 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.

3.5. Marking of bottles

a) 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 2). This may facilitate the cleaning of the bottles after the bioassay is completed;

b) Mark one bottle and its cap as control;

c) Mark the other four bottles and caps with the replicate number (1–4) and the bioassay date;

d) 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 and concentration;

Figure 2: Labeling bottles and caps.

e) Mark both the cap and the bottle so that bottles are associated with their respective caps. This is vitally important because the inside of the entire bottle will be coated, including the inside of the cap.

3.6. Bottle coating

a) Make sure that bottles and caps are completely dry;

b) Remove caps from the bottles;

c) If using disposable pipettes, label one pipette as 'solvent only' for the control bottle, and another pipette as 'insecticide solution' for the test bottles;

d) Add 1 ml of acetone/ethanol to the control bottle and put the cap back on tightly;

e) In the first test bottle, add a sufficient volume of the prepared insecticide stock solution to reach the desired diagnostic dose (Table 1). For example, if, as it was suggested, the stock solution has the same concentration of insecticide per ml as the diagnostic dose, add 1 ml of stock solution to the bottle. Put the cap back on tightly;

f) Repeat Step e with the other three test bottles;

g) Swirl the contents inside the bottle so that the bottom is coated (Figure 3);

h) Invert the bottle and swirl to coat the inside of the cap (Figure 4);

i) Place the bottle on its side for a moment to let the contents pool. Gently rotate while rocking the bottle gently so that the sides all the way around are coated (Figure 5);

Figure 3: Coating the bottom of the bottle.

Figure 4: Coating the top of the bottle.

Figure 5: Coating the sides of the bottle.

Figure 6: Drying the bottles.

j) Repeat this for all the test bottles;

k) Remove the caps and continue rolling bottles on their side until all visible signs of the liquid are gone from inside and the bottles are completely dry (Figure 6);

l) Leave bottles on their sides and cover with something that will keep them protected from light;

m) If bottles are not used right away, store bottles in a dark place (such as a drawer) with the caps off to avoid moisture build-up. If shipping pre-coated bottles, ship the bottles with the caps on. More information on the storage of coated bottles is given in Section 4.3.

4. CDC bottle bioassay method

4.1. General considerations

a) Use a filter in the aspirator to avoid inhaling mosquitoes or insect fragments;

b) Blow gently to expel the mosquitoes into the bottles. If you blow too hard, the mosquitoes can be damaged by hitting the sides of the bottle and killed before the insecticide has a chance to do so;

c) Be careful not to touch the inside of the bottle with the aspirator, as this may contaminate the aspirator;

d) Remember that the number of mosquitoes in the each of the test bottles does not need to be equal;

e) Determine species composition of mosquitoes either before or after the bioassay is conducted (Box 2).

4.2. 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:

a) Using an aspirator, introduce 10–25 mosquitoes into the control bottle. It is not necessary to count the mosquitoes; the exact number does not matter;

b) Introduce 10–25 mosquitoes into each test bottle; again, the exact number does not matter (Figure 7);

c) Start a timer. Be sure to examine the bottles at Time 0 and count the number of dead and/or live mosquitoes;

d) If you find dead mosquitoes at Time 0, make a note of them on the form (Appendix 3);

Figure 7: Transferring mosquitos into insecticide-coated bottles.

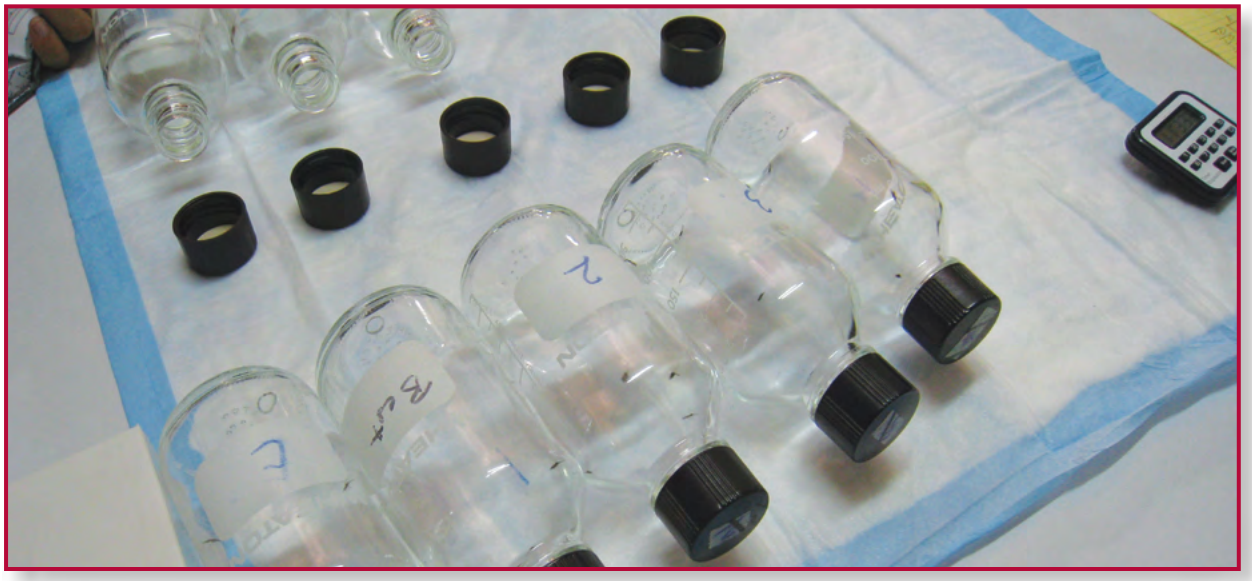


Figure 8: CDC bottle bioassay in progress.

e) Record how many mosquitoes are dead or alive, whichever is easier to count, every 15 minutes until all are dead, or up to 2 hours (Figure 8). It is not necessary to continue the bioassay beyond 2 hours;

f) Record these data on the reporting form (Appendix 3);

g) Graph the total percent mortality (Y axis) against time (X axis) for all replicates considered together using a linear scale;

h) Remember that mortality at diagnostic time is the most critical value because it represents the threshold between susceptibility and resistance. Refer to Table 1 for diagnostic doses and times for commonly used insecticides;

i) Take into consideration mortality in the control bottle at 2 hours (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%.

Mosquitoes are considered dead if they can no longer stand. See Box 3 for more information.

A timer could be started for each bottle, but it is sufficient to start one timer when the first or last bottle receives its mosquitoes. It is, however, important to be consistent and follow the same timer start procedure each time. Mosquitoes alive at the diagnostic time (Table 1) represent mosquitoes resistant to the insecticide being tested. These mosquitoes may be transferred to a sleeved carton for further analysis (e.g., molecular or biochemical assays). Mosquitoes flying at the end of the bioassay in the control bottle may need to be killed to get an accurate count. Mosquitoes can be killed by freezing or stunning them.

Box 3: Notes about mortality criteria.

- "Dead" mosquitoes are mosquitoes that cannot stand.
- It helps to gently rotate the bottle while taking the count.
- Immobile mosquitoes that slide along the curvature of the bottle can be easily categorized as dead.
- 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.

4.3. 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 (2–4 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. **It is prohibited to dry bottles in the oven after they have been coated with insecticide.**

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 off. Depending on the insecticide used, bottles can be stored from 12 hours to 5 days in this manner. The length of time bottles can be stored depends on the insecticide. Resmethrin- and Naled-coated bottles do not store well, so they should be used immediately after being prepared. Organophosphate-coated bottles should be used within 24 hours. To check if a stored bottle is still adequate, 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. Mosquitoes that survive the bioassay can be used for testing to identify mechanisms of resistance using enzymatic assays or molecular methods. Surviving mosquitoes may be easily released from bottles into a sleeved holding carton to separate them from those killed during the CDC bottle bioassay. Mosquitoes that will be further tested using enzymatic assays should be stored frozen. Mosquitoes to be used for molecular studies can be frozen, dried, or stored in 70% (or higher) ethanol. In addition, it may be necessary to use products like RNALater® (Applied Biosystems [Ambion], Foster City, California) to preserve samples for measurement of RNA levels associated with up-regulated enzyme mechanisms.

4.5. Validity of the data

With practice, the mortality of mosquitoes in the control bottle at 2 hours (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 at 2 hours, it is possible to either use Abbott's formula to correct the findings (see Box 4), 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 4: 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%)] x 100 = 44.4%

Note: In cases of 100% mortality in test bottles, Abbottt's formula has no effect. For example: [(100% - 10%) / (100% - 10%)] x 100 = 100% corrected mortality

4.6. Interpretation of results

As with other resistance bioassays, data from the CDC bottle bioassay using test mosquitoes need to be compared with data from susceptible mosquitoes or from a population that will serve as baseline. Resistance thresholds for each insecticide can be determined by calibrating the CDC bottle bioassay (Appendix 2). 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 9). 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 9, all mosquitoes that died before the diagnostic time when exposed to insecticide-coated bottles were susceptible. Test mosquitoes surviving beyond the diagnostic time threshold were assumed to have some degree of resistance. In the example, only 23% of the test population was susceptible. Recommendations for interpretation of bioassay data are shown in Box 5. The most important information is the mortality at the diagnostic time, but the bioassay is carried out beyond the diagnostic time to evaluate the intensity of resistance.

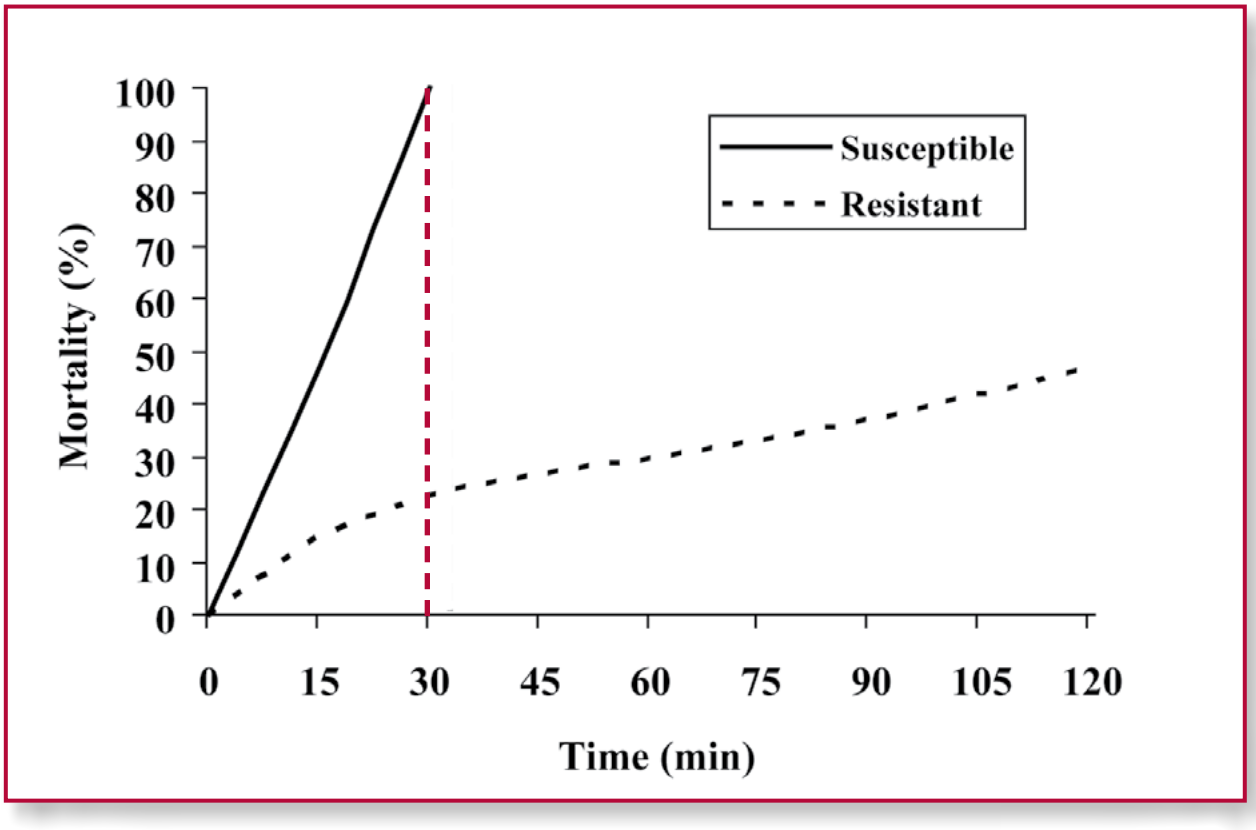


Figura 9: Determination of resistance threshold.

Box 5: Interpretation of data for management purposes.

WHO recommendations for assessing the significance of detected resistance:

- 98%–100% mortality at the recommended diagnostic time indicates susceptibility;
- 80%–97% mortality at the recommended diagnostic time suggests the possibility of resistance that needs to be confirmed;
- <80% mortality at the recommended diagnostic time suggests resistance.

Note: Where <95% mortality occurs at the diagnostic time in bioassays that have been conducted under optimum conditions and with a sample size of >100 mosquitoes, then resistance can be strongly suspected.

5. Resistance surveillance

5.1. Background

Although resistance data are often collected as part of vector control programs, this is often not done as regularly as it should be in a true resistance surveillance effort. Surveillance requires the regular collection and interpretation of epidemiological data to support changes in public health 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. Most malaria control programs carefully assess the efficacy of their vector control program by, for example, plotting incidence of malaria cases or by counting adult mosquitoes or larval collections at sentinel sites. The integration of insecticide resistance data and other kinds of malaria-related data needs to be taken into consideration before proposing and implementing remediation strategies for insecticide resistance.

5.2. 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 world. 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.

5.3. 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. In Guatemala, sampling sites for *Anopheles albimanus* only a few kilometers apart varied not only by presence or absence of resistance, but also by level of resistance and by dominant mechanism responsible for resistance. Generally speaking, 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.

5.4. 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.

5.5. 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 insecticide. 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. Areas 20 km 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 disease 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 operate sites for at least a few years, since comparative data are 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. This does not apply to situations such as the use of insecticide-treated bednets, where the insecticide exposure is year round. If several vectors in the area are seasonal, the resistance testing schedule should be adjusted to the species of interest.

It is also important to consider that it will be necessary to try to identify resistance mechanisms once resistance is detected with the CDC bottle bioassay, whether using the CDC bottle bioassay with synergists, or biochemical and/or molecular methods. Decisions on which insecticide to change to will depend upon the specific mechanism(s) of resistance.

Finally, some countries have found it useful to centralize preparation of bottles and administrative organization of surveillance. A central reference laboratory can provide support for technical assistance and quality assurance. It can also serve as a reference laboratory for training, provision of supplies, species identification, and enzymatic assays and molecular methods for determination of resistance mechanisms.

6. CDC bottle bioassay and synergists

6.1. Background

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

Once resistance is detected, or at least suspected, one must decide what to do next and which other compounds are likely to still be effective and not compromised by cross resistance. This requires knowledge of the resistance mechanism(s) in place, information usually acquired using either biochemical (microplate) assays or molecular methods. A rapid and inexpensive alternative to assess resistance mechanisms is to use the CDC bottle bioassay with synergists. Synergists are enzyme inhibitors of insecticide detoxification enzymes. Synergists are available for the metabolic detoxification enzymes: esterases, oxidases, and glutathione s-transferases.

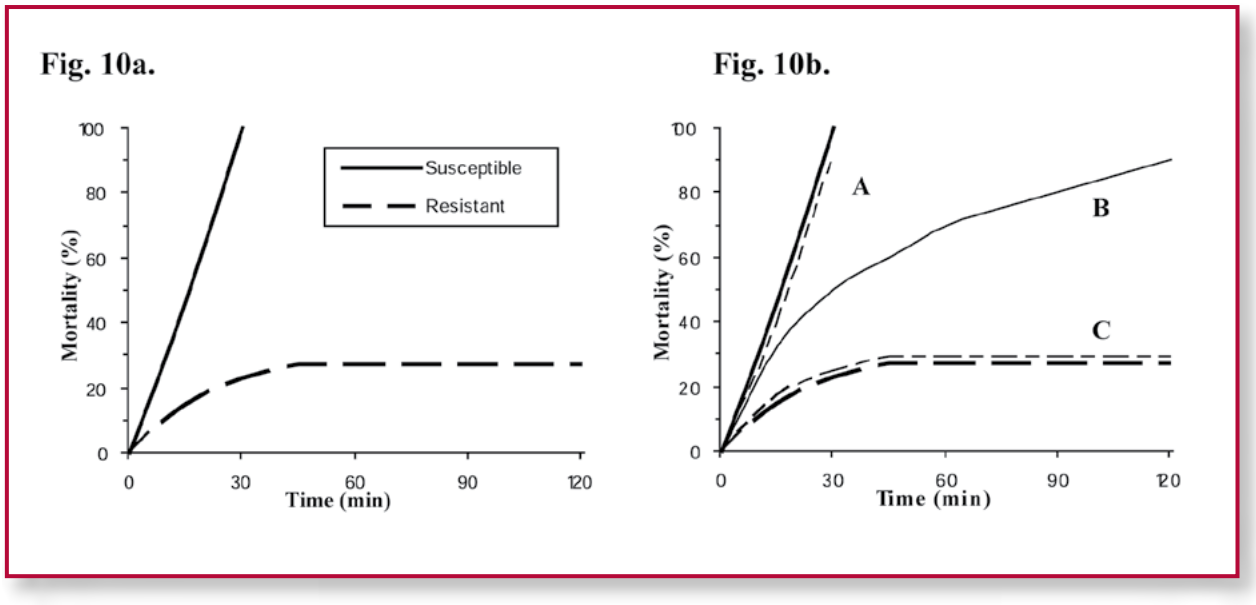
Synergists act by abolishing the apparent resistance observed in the CDC bottle bioassay if a detoxification enzyme plays a role in that particular resistance mechanism (Figures 10a and 10b). Data for resistant and susceptible populations are shown (Figure 10a). Once a synergist is used on the resistant 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 synergist 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 synergist 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 synergist is not involved in the resistance.

It is also possible to determine if a target site mechanism, such as the presence of the kdr gene (sodium channel mutation) or insensitive acetylcholinesterase, is involved. This is done by using the synergists in combination. Their combined use will not abolish the resistance in the bioassays when a target site mechanism is present. It is crucial in areas where pyrethroids and/or DDT are used to evaluate the relative role of detoxification and target site mechanisms involved in a particular incidence of resistance. A target site mechanism confers DDT–pyrethroid cross-resistance, while a detoxification mechanism may or may not. Knowledge of the resistance mechanism involved is required to select a replacement insecticide.



Figures 10a and 10b. Effects of synergists 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 synergist 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).

6.2. Use of synergists

Commonly used synergists in conjunction with the CDC bottle bioassay:

- Piperonyl butoxide (PBO), which inhibits oxidase activity;
- S.S.S-tributlyphosphorotrithioate (DEF), which inhibits esterase activity;
- Ethacrynic acid (EA), diethyl maleate (DM), and chlorfenethol (CF), which inhibit glutathione transferase activity.

It is also possible to use a combination of the above synergists. Testing mosquitoes with synergists is a two-step procedure. Mosquitoes are first exposed to the synergist(s) for 1 hour and then tested for insecticide resistance using the CDC bottle bioassay. A schematic representation of performing a bioassay with synergist(s) is shown in Figure 11.

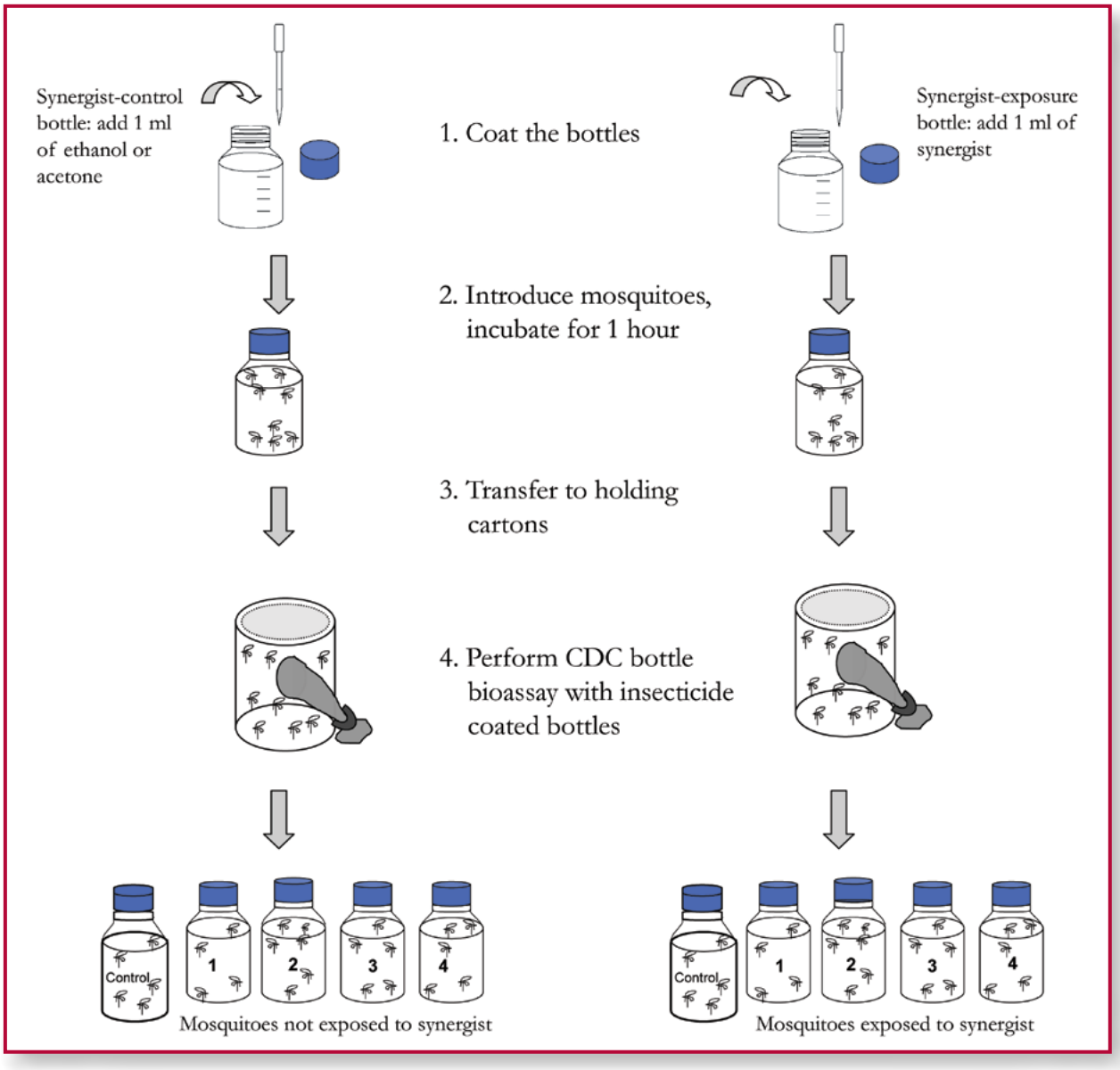


Figure 11. Performing the CDC bottle bioassay with synergists.

6.3. Preparation of bottles for synergist bioassays

To use the bioassay with synergists:

a) Prepare the synergist stock solution by diluting the appropriate amount of synergist in acetone or technical grade ethanol to be able to coat the bottles with the concentrations shown in Table 3. To make these stock solutions, use the same procedure used for making insecticide stock solutions (Section 3.2). In brief, dilute the appropriate amount of synergist in acetone or technical grade ethanol. To get a concentration of 400 µg/bottle of piperonyl butoxide, dissolve 400 mg in enough acetone or absolute ethanol to make 1 liter of solution. Each 1 ml of this solution will contain 400 µg of piperonyl butoxide;

b) Mark one bottle and its cap as the synergist-control bottle (without synergist);

c) Mark a second bottle and its cap to be the synergist-exposure bottle;

d) Add 1 ml of acetone or ethanol to the synergist-control bottle and put the cap back on tightly;

e) Add 1 ml of the synergist stock solution to the synergist-exposure bottle and put the cap on back tightly;

f) Coat the bottles, remove the caps, and let the bottles dry as in Section 3.6;

g) Prepare two test sets of bottles to run the CDC bottle bioassay (Section 3.6).

Table 3: Synergist concentrations used in the CDC bottle bioassay.

Synergist	Synergist concentration (µg/bottle)
Chlorfenethol	80
Diethyl maleate	80
Ethacrynic acid	80
Piperonyl butoxide	400
S.S.S-tributlyphosphorotrithioate	125

6.4. CDC bottle bioassay with synergist

To run the CDC bottle bioassay with synergists:

a) Introduce equal numbers of mosquitoes into the synergist-control bottle and into the synergist-exposure bottle (about 125 mosquitoes in each bottle);

b) Keep the mosquitoes in the bottles for 1 hour to allow the synergist to act;

c) After the 1-hour exposure is completed, transfer the mosquitoes to two holding cartons, one for the synergist-control mosquitoes and another for the synergist-exposed mosquitoes. This makes it easier to transfer mosquitoes into the insecticide-treated bottles;

d) Perform the CDC bottle bioassay as in Section 4.2 using one set of insecticide-coated bottles (one control and four test bottles) for the synergist-control mosquitoes and another set (one control and four test bottles) for the synergist-exposed mosquitoes;

e) Compare the data for the two populations of test mosquitoes.

6.5. Interpretation of bioassays with synergists

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

7. 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.

3. Zamora Perea E, Balta Leon R, Palomino Salcedo M, Brogdon W, Devine G, 2009. Adaptation and evaluation of the bottle assay for monitoring insecticide resistance in disease vector mosquitoes in the Peruvian Amazon. Malar. J. 8: 208.

APPENDIXES

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 (four coated and one control bottles), 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. Except in the case of organophosphate-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.

4. Can male mosquitoes be used for the control bottle?

No, males should not be used for the control bottles. Some resistance mechanisms are sex-linked, and one can be misled by using males in the control. In addition, most mosquitoes collected will be females.

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

Some people have found it useful to employ a piece of cotton wool held against the aspirating tube at the top of the bottle as the mosquitoes are being introduced into the bottles. As the aspirator is withdrawn after the mosquitoes are introduced, the cotton wool can be used to close the bottle top, until the bottle cap is put in place. In our experience, a swift decisive puff of air will introduce mosquitoes without loss. Attempting to introduce mosquitoes into a bottle more than once may allow some to escape. This sometimes happens if the user attempts to put exactly the same number of mosquitoes into each bottle, which is not necessary.

6. 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 two ways that this can be dealt with. First, mosquitoes can be randomly selected. Alternatively, mosquitoes can be held for one or two days for the blood meal to be digested and then used for the bioassay.

Appendix 2. Diagnostic doses and CDC bottle bioassay calibration

It is assumed that resistance is present if a diagnostic dose, proven and validated against a susceptible insect population, is survived by members of a test population at a predetermined diagnostic time. The diagnostic dose and diagnostic time are optimal parameters for detecting insecticide resistance. A diagnostic dose that is too low will not kill susceptible mosquitoes during the bioassay, providing a false-positive result for resistance. On the other hand, a diagnostic dose that is too high will kill resistant mosquitoes during the bioassay, masking resistance.

For some insect vectors from some geographic regions, diagnostic doses and diagnostic times for several insecticides have already been determined. It is recommended that countries in these regions use the already established parameters to allow them to compare data across countries or within regions. However, if this information is not available, the diagnostic dose and the diagnostic time will need to be defined for each insecticide, in each region, and for each main vector species that is to be monitored. To determine the diagnostic dose and the diagnostic time for use in the CDC bottle bioassay, the assay will have to be calibrated.

Calibration assay

The assay is calibrated by first selecting the testing population and possible lengths of test time, and then by determining possible diagnostic doses, given preferred diagnostic times.

Population: The first step is to select a susceptible vector population to use as a baseline. If such a population is not available, it is possible to use the vector population from the area where the chemical vector control measures are to be applied. This will be the reference point against which all future populations can be compared.

Diagnostic time: For practical reasons, the diagnostic time should be between 30 and 60 minutes.

Diagnostic dose: The diagnostic dose will be a dose of insecticide that can kill 100% of mosquitoes sometime between 30 and 60 minutes and that is below the saturation point. To determine possible diagnostic doses, first prepare bottles with a range of different concentrations of insecticides per bottle, as outlined in the guideline. Using each of these different bottles, run separate CDC bottle bioassays on 25 mosquitoes of the susceptible population to determine the upper limit of the diagnostic dose, which is the saturation point. The saturation point can be defined as a concentration above which the time to kill 100% of the mosquitoes remains the same even if the concentration increases. See a more detailed explanation on how to determine the saturation point below.

It may be necessary to run additional sets of concentrations around that range until the optimal diagnostic dose is determined. For example, starting with 10 µg/bottle, increase concentration with increments of 5 µg and continue to a final concentration of 200 µg/bottle. If no clear saturation point can be determined, run more assays using bottles with <10 µg/bottle and/or >200 µg/bottle, with increments of 5 µg. If the saturation point still cannot be determined, more assays may be run with bottles using smaller increments of insecticide.

Interpretation of calibration data

Graphing the results of the calibration assay will show that the time-mortality line becomes straighter, steeper, and closer to the Y-axis as the insecticide concentration increases (Figure). This means that by increasing insecticide concentration the time-mortality line will reach a point where increasing the concentration of insecticide will not kill all mosquitoes any faster. In the example below, 15 µg/bottle is the toxicological saturation point for insecticide entering the mosquito and reaching its target. Increasing the concentration to 25 µg/bottle does not cause the insecticide to penetrate the mosquito, reach the target site, and kill the mosquito any faster. Therefore, 15 µg/bottle is the saturation point and the maximum concentration to use as the diagnostic dose. Otherwise, there is a risk that resistant mosquitoes will be killed by doses higher than the saturation point and then be recorded as susceptible, i.e., false negatives for resistance.

A slightly smaller concentration compared to the toxic saturation point will kill mosquitoes in an amount of time perhaps more convenient for the user (e.g., 30 to 60 minutes). So, it is possible to choose a lower diagnostic dose that kills 100% of mosquitoes within 30 to 60 min. It must be understood that several different pairs of diagnostic doses and diagnostic times will give interpretable results, but it is necessary to consistently use the same diagnostic dose and diagnostic time for that particular insecticide on that vector in future assays over long periods of time to allow for comparability. Otherwise, the method will not allow assessment of changes in resistance over time for that species.

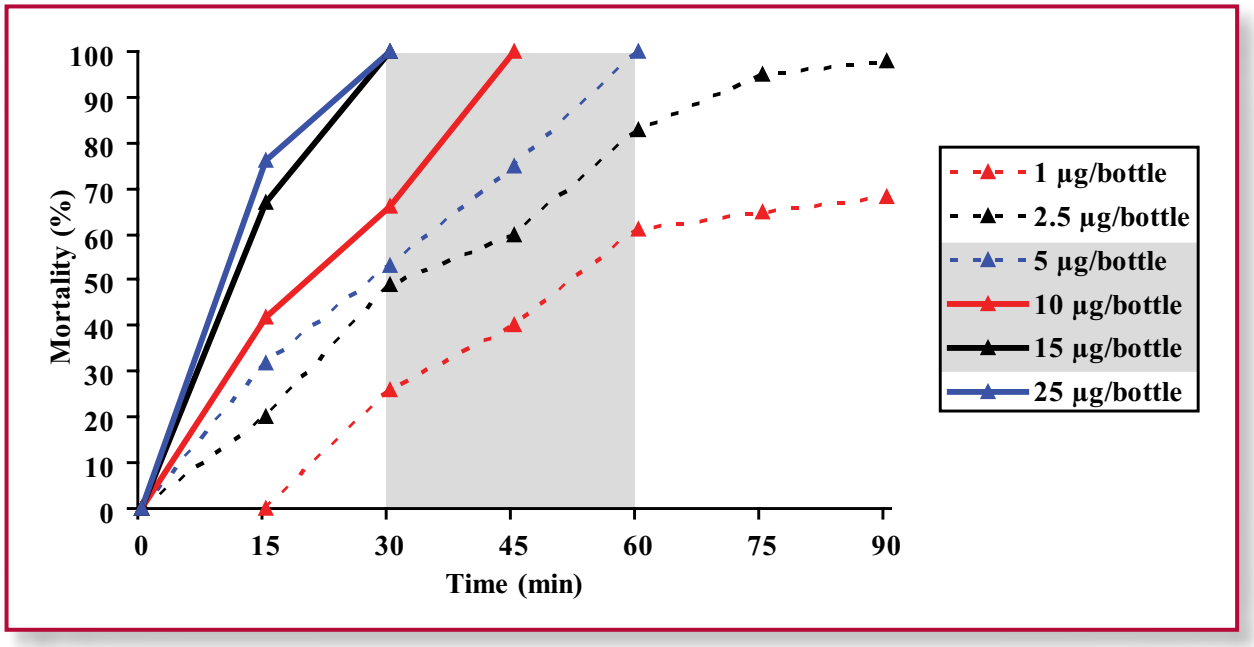


Figure: Determining diagnostic doses and diagnostic times.

In the example shown, 15 µg/bottle is the saturation point because higher doses did not decrease the time for 100% of susceptible mosquitoes to be killed. Concentrations <5 µg/bottle take more than 60 minutes to kill 100% of susceptible mosquitoes, which means that mosquitoes would take a long time to be killed by these doses. The doses between 5 and 15 µg/bottle (shaded area) are in the usable range for detecting resistance, and the diagnostic time for each of these concentrations will be the time at which 100% of mosquitoes were killed. So, for example, a diagnostic dose of 10 µg/bottle and diagnostic time of 45 min could be selected.

Appendix 3. CDC bottle bioassay data recording form

Date: _____ Mosquito species: _____

Insecticide: _____

Diagnostic dose: _____ Diagnostic time: _____

Location of mosquito collection: _____

Time (min)	Bottle 1		Bottle 2		Bottle 3		Bottle 4		All test bottles			Control		
	Alive	Dead	Alive	Dead	Alive	Dead	Alive	Dead	Total dead	Total	% dead	Total dead	Total	% dead
0														
15														
30														
35														
40														
45														
60														
75														
90														
105														
120														
Total in bottle														