Chemline

# The Bottle Bioassay for Measuring Resistance

by *Janet McAllister and William Brogdon*

Concern over the development of resistance to insecticides in public health arthropods has increased in recent years. This renewed concern was been heightened by the fact that in vector control there are only a few classes of compounds available as "tools of the trade." The loss of any of these tools for any reason could seriously impact our ability to keep our mosquito and fly populations at tolerable levels. Dr. David Dame wrote an excellent review of how resistance develops and how it can be managed in the last issue of *Wing Beats* so this article will concentrate on how to test for resistance. While some think testing for resistance is some complicated laboratory endeavor, this is simply not true. While there is a lot of confusion over what resistance is or is not, the bottom line is that reduced control, or even a failure of control, may be due to many factors besides insecticide resistance. Determining if it's you or your mosquitoes is something every district can do.

There is a new technique, or rather an old idea in a new format. The bottle bioassay is easy to use and gives some indication of what mechanism is involved if resistance is discovered. The idea of using time mortality for assessing the susceptibility of a population is a basic concept in toxicology. The adaptation of this idea into a technique that is simple to use even in the field under poor conditions is relatively new in mosquito control. The bottle can replace other more time consuming and complex bioassay methods, such as the WHO field test, topical applications to determine dose mortality and wind tunnel testing. Its advantages are that it is 1) quick, results can be generated in 2 to 3 hours; 2) can be prepared any place at any time using any insecticide; 3) the results are reproducible without needing huge numbers of mosquitoes and replications and 4) the results can be correlated with other tests for determining what mechanism is acting to confer resistance.


A little background is needed to understand what the bottle bioassay is measuring. When an insecticide comes in contact with a mosquito or other insect, it must penetrate the cuticle, traverse intervening tissue to get to its target site, and perhaps even be metabolized into a different "active form" along the way. It takes a little while for the mosquito to get sick and die. This process takes a certain amount of time in susceptible mosquitoes. Anything that the mosquito has developed to interfere with or delay this process is resistance. The bottle bioassay is a quick and easy way to determine if your mosquitoes have developed anything to delay or avoid getting sick and dying.

## Running Bottles

How do you run a bottle bioassay? Coat the inside of a glass bottle with the insecticide you are interested in, put some mosquitoes into the bottle and at timed intervals count how many mosquitoes are dead. You then look at these data to see if your mosquitoes are taking longer to die than a susceptible population. If they are, you can run the whole test again, this time including some synergists which will help determine why the mosquitoes are taking longer to die.

Now, lets walk through the process one step at a time in a little more detail. To coat the bottle, you put 1 to 1.5 ml of acetone in the bottle and add technical grade insecticide. The amount of acetone is not critical because you are using it to spread out your insecticide. This is not a dilution because the acetone will evaporate, leaving the insecticide behind. The amount of insecticide you use will be on the order of 25 to 800 micrograms per bottle depending on the chemical. This is not a very large amount of insecticide; to make things easier it is convenient to dilute technical insecticide into a stock solution. You will add insecticide to the bottles from this stock solution. Once everything is in the bottle, you cap it and roll it around to coat the entire surface inside, including the cap. The next step is to uncap the bottle and allow the acetone to completely evaporate. It is important to continue rolling the bottle while the acetone evaporates to make sure the insecticide is spread on all surfaces and not just on the bottom of the bottle. The bottle and its cap are treated as one unit and should be kept together. Be sure to run some control bottles that were treated with acetone, but no insecticide. You can run more than one batch of mosquitoes in a set of bottles. Just make sure that you don't get moisture build up from blowing mosquitoes into the bottles. When you are done with the bottles they can be reused by cleaning them properly. To do this, triple-rinse them with acetone and wash them with soap and water.

After your bottles have completely dried they are ready for mosquitoes. Add the mosquitoes all at once to each bottle. You won't have time to add them one at a time and they will escape from the bottle if you keep taking the cap off. Check to make sure you didn't kill any while putting them in the bottle. If you did, make a note of it and subtract them from your data. The number of mosquitoes you put in a bottle is not critical. Use as many mosquitoes as you can easily keep track of. As you get familiar with running bottles you will be able to increase the number you put in each bottle. Count the number dead or alive, which ever is easier for you, at timed intervals. The amount of time you wait between readings is not critical. We find 15 minutes to be a good time. If you are working with a faster acting insecticide you may want to reduce the time between readings to every 10 minutes. When you stop the test you can count the total number of mosquitoes so you can go back and calculate the percent mortality at each reading.

## Calibration

How much insecticide do you use? Well, you calibrate your bottles to determine this amount by doing some pre-testing. By running several sets of bottles with different concentrations you can determine two important parameters of the bottle test, the saturation point of susceptible mosquitoes for a particular insecticide and a time threshold for susceptibility. As you increase the amount of insecticide in your bottles, you will find the mosquitoes die quicker. Eventually a concentration will be reached where the insecticide can not get into the mosquito and kill it any quicker, no matter how much you increase the amount of insecticide. This concentration is the saturation point. Once you know this saturation point, you can make your bottles using the lowest concentration where saturation was achieved. A list of concentrations we use for 250 ml glass bottles is in Table 1. You don't have to calibrate your bottles every time you run tests. Once they are calibrated, you know how much to put in each time you make bottles. The second parameter you have just determined is the time threshold for susceptibility: the time it took to kill 100% of your mosquitoes. This is the parameter you will use to determine the proportion of your mosquitoes with resistance. Susceptible mosquitoes will die in a certain amount of time, dependent on the species. The mosquitoes that survive longer than this threshold time are the ones that have something in them making them resistant to the insecticide.

**Table 1. Stock solutions and bottle dosages currently used at the CDC.**

| Stock (Techgrade AI + Acetone) | | Amount of Stock added to 250 ml bottle | Final concentration/bottle* |
|---|---|---|---|
| | | **Insecticides** | |
| Permethrin | 4.3 µl/ml | 10 µl | 43 µg/bottle |
| Resmethrin | 10 µl/ml | 3 µl | 30 µg/bottle |
| Naled | 10 µl/ml | 2.5 µl | 25 µg/bottle |
| Malathion | 10 µl/ml | 40 µl | 400 µg/bottle |
| Fenthion | 10 µl/ml | 80 µl | 800 µg/bottle |
| Temephos | 10 µl/ml | 85 µl | 850 µg/bottle |
| | | **Synergists** | |
| DEF | 50 µl/ml | 2.5 µl | 125 µg/bottle |
| PBO | 4 µl/ml | 100 µl | 400 µg/bottle |
| **Control** | | 1 ml | 0 µg /bottle |

**Final concentrations are based on a density of 1 for the technical grade insecticide. If insecticide is purchased as a solid instead of a liquid, then the proper amount can be weighed to come to a final concentration in µg/bottle.*

## Using bottles to determine mechanisms

One of the nifty things about the bottle bioassay is that you can also easily combine chemicals. If you find resistance to a particular insecticide, you can run some more bottles with the insecticide plus a synergist. This will give you a clue as to what mechanism your mosquitoes have developed to survive.

There are several ways that insects avoid the toxic effects of insecticides. One way is to produce large amounts of enzymes that overwhelm the insecticide. In general, the two most important of these enzymes for mosquito control in the U.S. fall into one of two classes of proteins, the oxidases and the esterases. Oxidases usually are the enzymes involved in pyrethroid resistance and esterases are usually involved in organophosphate resistance. Oxidases are synergized by PBO (piperonyl butoxide) and esterases are synergized by DEF (S.S.S tributlyphosphoro-trithioate). In other words, these two chemicals block the effects of one or the other

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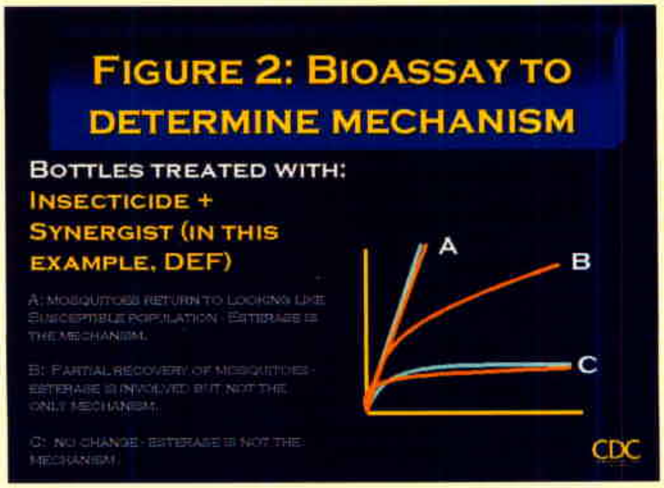
of the two most important detoxification enzymes. If there are high levels of one of these enzymes present in a resistant population then addition of one of these synergists should make the population look susceptible again.

So if you add PBO to the bottles with an insecticide for which you saw resistance and the same population is killed




quickly like a susceptible population, then you can infer that high levels of oxidase are present. The same is true for DEF and esterases (Figure 2).

You can also have another scenario. You can have cases where one or both synergist only partially abolishes the effects of resistance. This tells you that more than one mechanism is acting on the population; this is called multiple resistance. There are many cases where both enzyme families contribute to resistance in the same insects.

Another way insects avoid the toxic effects of insecticides is to change the target site where the insecticide acts. If this is the mechanism your mosquitoes have developed you will see no change in your bottle bioassay results when you add one of these synergists. You will have to perform more tests to determine the mechanism of resistance.

As you can see, this assay is easy to use and can give you a lot of meaningful information about your mosquitoes. The true value of the bottle bioassay is it allows you to collect data in real time on a particular population, at a particular time, in a particular place. This "snapshot" of susceptibility can be used to make a decision on the spot as to what chemical to use (or avoid because of resistance) or it can be compared with previous "snapshots" to give you an idea of how your mosquitoes are changing over time in the face of selection through insecticide exposure.

**Janet McAllister is a Visiting Fellow and William Brogdon is a Research Entomologist with the Division of Parasitic Diseases at the Centers for Disease Control and Prevention in Atlanta, GA.**