



I2I Landscaping exercise

CDC bottle bioassay

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Acronym List

AI	Active ingredient
CDC	Centre for Disease Control
DDT	Dichlorodiphenyltrichloroethane
LSTM	Liverpool School of Tropical Medicine
MoA	Mode of action
PPF	Pyriproxyfen
SOP	Standard operating procedure
WHO	World Health Organisation

Summary

Aim and key questions addressed	<ul style="list-style-type: none"> - Surveillance tool for detecting resistance in vector populations - Mosquitoes are exposed to pre-determined concentrations of insecticides, allowing resistance to be detected
Context	<ul style="list-style-type: none"> - Laboratory
Test item	<ul style="list-style-type: none"> - Mosquito colony
Mosquito population	<ul style="list-style-type: none"> - Laboratory reared and wild populations
Number of mosquitoes per replicate	<ul style="list-style-type: none"> - Minimum 100, over four replicate bottles
Endpoints measured	<ul style="list-style-type: none"> - 24-hour mortality
Exposure time	<ul style="list-style-type: none"> - See relevant protocol for active ingredient tested
Holding time	<ul style="list-style-type: none"> - See relevant protocol for active ingredient tested
Indicative of personal protection	<ul style="list-style-type: none"> - No
Suitable chemistries	<ul style="list-style-type: none"> - Chemistries applied to glass surfaces
Appropriate controls	<ul style="list-style-type: none"> - Negative control: exposure bottle with suspension oil only
Relevant stage of production pipeline	<ul style="list-style-type: none"> - Mosquito characterisation

Characterisation of output	<ul style="list-style-type: none"> - Endpoints for pyrethroids and pyrethroid synergists are well defined. Adaptation is needed for active ingredients with different endpoints, such as pyriproxyfen.
Accessibility	<ul style="list-style-type: none"> - Materials and set-up are easily accessible
Cost	<ul style="list-style-type: none"> - Low
Level of validation and characterisation of outputs	<ul style="list-style-type: none"> - Validation of this method is unclear. This method is reported as being used frequently in the published literature, however there are no published multicenter studies of this method.
Outstanding questions, gaps and priorities	<ul style="list-style-type: none"> - Lack of clarity based on published studies as to whether methodological variations influence mortality data. - These potential sources of variation need to be researched through a series of bottle bioassays.
Key references, related SOPs, guidelines and publications	<ul style="list-style-type: none"> - Brogdon, W., & Chan, A. (2010). Guideline for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle Bioassay. 1–28. - Enhanced Surveillance Protocol for the CDC Intensity Bottle Bioassay. (2013).

Overview

The Centers for Disease Control and Prevention (CDC) bottle bioassay was developed by Brogdon and McAllister in 1998 and updated in 2010 (Brogdon & Chan, 2010). It is a surveillance tool for resistance detection among malaria vectors, which looks at time-to-kill of a population exposed to a known concentration. The CDC bottle bioassay relies on time mortality data, which are measures of the time it takes an insecticide to penetrate a vector, reach the target site and act on the site. Information derived from this bioassay may provide initial evidence that an insecticide is losing its effectiveness. Briefly, the CDC bottle bioassay method comprises of introducing mosquitoes into glass bottles that have been coated with a known concentration of insecticide and exposing them for a set length of time. Mosquitoes are recorded as dead or alive at the end of the assay.

The data generated is widely used for routine resistance monitoring of field and laboratory mosquito populations. Advantages to this assay include the ability to screen a range of concentrations of active ingredient, as well as testing synergists for assessment of metabolic resistance mechanisms. Reliable data from this assay is of great importance, problematically there are high levels of inconsistencies in reported data (Strode *et al.*, 2014). Additionally, data available from the CDC bottle test conducted at the Liverpool School of Tropical Medicine (LSTM) shows variation between replicates carried out with the same compounds/concentration

Define Accepted Methodologies

Are there existing standard SOPs/Guidelines detailing methodologies?

Despite the CDC outlining guidelines for the CDC bottle bioassay test procedure, these are not a standard operating procedure (SOP), therefore leaving room for discretion.

- Guideline for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle Bioassay (Brogdon & Chan, 2010)

- Enhanced Surveillance Protocol for the CDC Intensity Bottle Bioassay (insert for guidelines (*Enhanced Surveillance Protocol for the CDC Intensity Bottle Bioassay*, 2013)

There is also a World Health Organisation (WHO) modified version of the CDC bottle bioassay:

- Standard operating procedure for testing insecticide susceptibility of adult mosquitoes in WHO bottle bioassays (WHO., 2022).

Are these sufficiently detailed?

The general method is quite vague leaving room for interpretation and subsequently causing inconsistencies.

Do these methods require specialised/non-standardised equipment and/or training?

The methods do not require specialised equipment. However, specific training on preparing insecticide dilutions, coating bottles, mosquito handling and conducting the assay is required, with the suggestion that variation from uses may lead to variability in results.

Are there issues with the methods or their interpretation?

As the method outlined is vague there is room for inconsistencies (outlined below).

What AIs or combinations of AIs have the tests been used for?

Many active ingredients (AI) have been tested. Commonly assessed AIs include Bendiocarb, Cyfluthrin, Cypermethrin, DDT, Deltamethrin, Fenitrothion, Lambdacyhalothrin, malathion, permethrin, pirimiphos-methyl. The CDC bottle assay is often used to establish discriminating doses for novel or repurposed AIs for vector control. Commonly, this method would expose a mosquito strain(s) to a range of doses of the test AI and report relevant endpoints (e.g., knockdown or mortality) depending on the mode of action (MoA) of the AI.

Are they validated, for which AIs/entomological effects, and to what extent?

Validation of this method is unclear. This method is reported as being used frequently in the published literature, however there are no published multicenter studies of this method.

What inputs need to be characterised? e.g., samples, mosquitoes, equipment

- Horizontal/vertical orientation of bottle - CDC guidelines provided for the performance of the assay also specify that the bioassay can be conducted upright or sideways. This lack of consistency between testing is potentially problematic as previous research has demonstrated that the angle at which testing is performed influences contact with a treated surface and thus mortality (Owusu & Muller, 2016). The angle of the bottle bioassay might, therefore, influence the likelihood of resting behaviour versus flight and cause fluctuations in contact with the treated sides dependent upon the bottle orientation.
- Number of mosquitoes per test unit - a higher quantity of mosquitoes per bottle may cause disturbance to resting mosquitoes, increasing flight activity. CDC bottle guidelines state that between 10-25 mosquitoes can be used per assay (Brogdon & Chan, 2010).
- The age of mosquitoes.
- Knowledge gaps exist in mosquito behavioural characteristics during testing in the CDC bottle bioassay. Insecticide delivery relies on mosquitoes contacting the bottles treated surface. How mosquitoes interact with the surfaces has never been fully elucidated or quantified. Flight and resting behaviour both influence contact with the surface, variations in these could result in fluctuations in the mortality data generated.
- In addition to quantifying resting behaviour on a surface, it is also important to define the specific areas that this occurs in the bottle. Coating coverage could differ based on human variability. Prolonged or frequent contact with a surface that has not been evenly coated with the insecticide could fluctuate mortality results.
- Bottle agitated/still - Key behaviours could also be disturbed by agitation to the bottle by the user during testing.
- Temperature/humidity of the testing room during bioassay

- The method of reporting mosquito mortality differs between publications – some users report mortality every 15-minutes, whereas others report at 30-minutes. A clearer breakdown of time points to record mortality at would be useful to enhance the precision when comparing diagnostic doses between studies.

Are endpoints clearly defined and appropriate? Who were they defined by?

Knock down and mortality endpoints are clear and appropriate for certain AIs. Adaption and addition will be required for AIs whose MoA differ from fast acting mortality (e.g., pyriproxyfen [PPF] causes female sterilization).

Are their supporting SOPs? e.g., cleaning SOPs, mosquito rearing SOPs required.

Three cleaning SOPs were suggested as part of the WHO multicenter study for the cleaning of Wheaton bottles. These protocols are not validated and are outlined below:

LSTM washing method:

1. Take approximately 10 ml of acetone and add it to a bottle.
2. Shake vigorously.
3. Transfer the acetone to other bottle.
4. Shake vigorously.
5. Repeat 7.1-7.4 with all bottles.
6. Take another 10 ml of acetone.
7. Repeat steps 7.1-7.5
8. Discard the acetone.
9. Prepare a 2-5 % DECON solution in hot water.
10. Using a cleaning brush, scrub vigorously every bottle with the DECON solution.
11. Wash the soap with tap water.
12. Prepare a 10-litre container with clean water.
13. Submerge the clean bottles.
14. Leave them there for 24 hours.
15. Dry the bottles overnight or in an oven (50°C) for 15-20 minutes.

Note: same insecticide impregnated bottle can be used three times for assays, but it is necessary to leave them unused 2-4 hours between assays. Between assays, the bottles should be stored at 4°C in the dark. After three uses or five days after impregnation, the bottles need to be washed.

CDC washing method

1. Fill sink $\frac{3}{4}$ with hot water.
2. Add 150ml of soap solution.
3. Soak for a minimum of 15 min.
4. Scrub once with brush.
5. Empty bottle.
6. Refill with soapy water.
7. Continue until all bottles are done.
8. Drain sink.
9. Take out bottles.
10. Triple rinse bottles and caps with hot water.
11. Use washing machine.
12. Autoclave when completed.
13. Rinse caps with acetone before use.

IRD washing method (adapted from LSTM):

1. Take approximately 10 ml of acetone and add it to each bottle.
2. Shake vigorously.
3. Discard the acetone.
4. Prepare a 20% DECON solution in hot water in 20L container (15L = 15 bottles).
5. Submerge bottles in DECON 20% overnight.
6. The next day, remove DECON 20% from each bottle.
7. Wash the DECON 20% three times with tap water.
8. Submerge the clean bottles all day in tap water.
9. At the end of the day, dry the bottles upside down overnight (as Figure 3) or in an oven (50°C) for 15-20 minutes.



Figure 1 - Dry the bottles upside down raised by a support

Define Current Use Practices

Does everybody use the same SOP?

The majority of publications reference the 2010 guidelines (Brogdon & Chan, 2010).

Are there differences of interpretation of the method?

Where inputs are not specified this is left up to the user to decide.

- Horizontal / vertical orientation of the bottle
- Sample size
- Number of mosquitoes per test unit
- Age of mosquitoes
- Units ($\mu\text{g}/\text{bottle}$ and $\mu\text{g}/\text{mL}$)

Are there results obtained largely consistent between studies?

Studies often test different concentrations of AI or use different mosquito strains, so comparability is difficult. There are also questions around reporting of mortality at different time points which can make comparison more problematic.

Is further development, refinement or validation of the method required? Based on priority, significance, and relevance of method.

Some optimisation and refinement are underway as part of I2I review.

Identify Potential Sources of Variation

What are the sources of variability in the method and are their means to minimise or characterise these.

Despite CDC outlining guidelines for the CDC bottle bioassay test procedure, these are not to a standard operating procedure, therefore leaving room for discretion (CDC, 2010). Bottles are also self-prepared by the user with insecticides being coated by hand, which is a potential source of variation and less consistent in comparison to the WHO tube assay which uses insecticide-impregnated papers all acquired from a central source. Methodological variations may have arisen between users during testing.

There is a variability experiment underway with I2I to assess sources and quantify the variability within this method.

Do current method/s need to be adapted for new active ingredients/MoA/types of tool?

Currently adapted for use with chlorfenapyr and pyriproxyfen as part of the WHO multicenter study.

Are new methods required? Identify areas where current method/s are not suitable or sufficient.

A comparison between the CDC bottle bioassay (Brogdon & Chan, 2010) and the WHO bottle bioassay (WHO, 2022) could help with identifying which method is more informative.

Gaps in biological or other understanding that hinder method development or validation

- More work is needed to understand the use of adjuvants and consistency of bottle coating, which is problematic due to the difficulty in chemically analyzing treated bottle surfaces.
- Knowledge gaps exist in mosquito behavioural characteristics during testing in the CDC bottle bioassay. Insecticide delivery relies on mosquitoes contacting the bottles treated surface, but how mosquitoes interact with the surface has never been fully elucidated or quantified.

Prioritisation – is there an issue that needs to be addressed, what specifics, how urgent is the need?

Due to a lack of clarity based on published studies as to whether methodological variations influence mortality data, these potential sources of variation need to be researched through a series of bottle bioassays.

A piece of work performed by Althoff & Huijben (Althoff & Huijben, 2022) compared the variability in mortality data generated by CDC bottle bioassay, WHO tube test and topical application bioassay using *Aedes aegypti* mosquitoes.

- Results showed that the topical application bioassay exhibited the lowest amount of variation in the dose-response data, followed by the WHO tube test. The CDC bottle

bioassay had the highest level of variation. In the fixed-dose experiment, a higher variance was similarly found for the CDC bottle bioassay compared with the WHO tube test and topical application.

References

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