I2I Landscaping exercise: WHO bottle bioassay.

July 2023

Annabel Murphy

Acronym List

AI	Active ingredient
CDC	Centers for Disease Control
DC	Discriminating concentration
LC_{50}	Concentration that kills 50% of the test population
LC_{95}	Concentration that kills 99% of the test population
MoA	Mode of action
OI_{50}	Concentration that inhibits oviposition of the test population by 50%
OI_{99}	Concentration that inhibits oviposition of the test population by 99%
PPF	Pyriproxyfen
SOP	Standard operating procedure
WHO	World Health Organisation

Overview

The World Health Organization (WHO) bottle bioassay is used to evaluate the susceptibility of adult mosquito vectors to insecticides. It is a modified version of the Centers for Disease Control and Prevention (CDC) bottle bioassay (Brogdon & Chan, 2010) using endpoints similar to those of the current WHO tube test to enable mosquito mortality to be evaluated at the same time point post exposure (World Health Organisation, 2021) and to enable evaluation of insecticides that are not suitable for impregnation onto Whatman no.1 filter papers (WHO, 2022).

Define Accepted Methodologies

Are there existing standard SOPs/Guidelines detailing methodologies?

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

Are these sufficiently detailed?

Most of the methods are sufficiently detailed, however there is still room for inconsistencies. Coating coverage of the insecticide onto the bottle surface is done by hand and can differ based on human variability. Additionally, the flight and resting behaviour of the mosquito will influence contact with the insecticide.

Methods that are detailed sufficiently in comparison to the CDC bottle bioassay include:

- Number of mosquitoes per test unit - the guidelines for the WHO bottle bioassay state to use 25 mosquitoes, or as close to 25 mosquitoes as possible. CDC bottle guidelines state that between 10-25 mosquitoes can be used per assay (Brogdon & Chan, 2010). The number of mosquitoes per bottle can affect resting behaviour and therefore insecticide exposure.
- Orientation of the bottle during testing-the WHO bottle bioassay guidelines state to keep the bottle vertical during the 1-hour exposure period. The CDC guidelines in comparison

specify that the bioassay can be conducted upright or sideways. The angle of the bottle bioassay might, influence the likelihood of resting behaviour versus flight and cause fluctuations in contact with the treated sides dependent upon the bottle orientation, previous research has demonstrated that the angle at which testing is performed influences contact with a treated surface and thus mortality (Owusu & Muller, 2016).

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

Specific training is required for preparing insecticide dilutions, coating the bottles, mosquito rearing and preparation and carrying out the assay. No specialised equipment is required.

Are there issues with the methods or their interpretation?

Knowledge gaps exist in mosquito behavioural characteristics during testing in the WHO bottle bioassay. Insecticide delivery relies on mosquitoes contacting the treated surface of the bottle. How mosquitoes interact with the surface 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. Although there is a detailed procedure and diagrams in the WHO bottle procedure, coating coverage could still 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.

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

Active ingredients (AI) that have been tested in (Govoetchan et al., 2023) and (Corbel et al., 2023) are:

- Pyrethroids: metofluthrin, prallethrin and transfluthrin
- Neonicotinoids: Clothianidin
- Pyrroles: Chlorfenapyr
- Juvenile hormone mimics: pyriproxyfen (PPF)
- Butenolides: flupyradifurone
- Broflanilide (trade name TENEBENAL)

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

(Corbel et al., 2023) conducted a multi-center study which aimed to validate the WHO glass bottle bioassay method as an alternative to the WHO standard insecticide tube test. The multi-center study tested over 200,000 mosquitoes and involved 21 worldwide laboratories to obtain data on the susceptibility of the mosquito species: *Aedes aegypti, Aedes albopictus, Anopheles gambiae* sensu stricto, *Anopheles funestus*, *Anopheles stephensi, Anopheles minuimus* and *Anopheles albimanus* to seven insecticides: metofluthrin, prallethrin, transfluthrin, clothianidin, chlorfenapyr, pyriproxyfen and flupyradifurone in glass bottle assays.

LC_{50}, LC_{99} (concentration that kills 50% or 90% of the test population), or OI_{50}, OI_{99} (concentration that inhibits oviposition of the test population by 50% and 99%) results were obtained and the results showed that the mean within-bioassay variability in mortality and oviposition inhibition were <10% for most mosquito species-insecticide combinations. Variation occurred between laboratories for some species-insecticide combinations however this more commonly occurred specifically with transfluthrin and flupyradifurone. The conclusions/findings from this study have been used by the WHO to establish 17 new insecticide discriminating concentrations (DCs) for *Aedes/Anopheles* spp.

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

The equipment required is well detailed in the SOP. The SOP details that the bioassay requires 150 non-blood-fed adult female mosquitoes aged 3-5 days before testing. The overall sample size is dependent on the study and the mosquito-insecticide combinations are determined by the study protocol.

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

The endpoints are clearly defined in the SOP (WHO, 2022) with mosquito mortality being measured 1 hour after exposure to a discriminating concentration of insecticide and the mortality again recorded at 24 or 72 hours later. Adaptations are required depending on the type of AI used, where mode of action may differ, for example with pyriproxyfen (PPF), a juvenile hormone mimic with sterilizing properties.

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

The SOP provided by (WHO, 2022) outlines 2 different options for washing the bottles, depending on the availability of washing agents.

Generic wash method using Decon or TFD4

Note: TFD4 solution is an alkaline detergent consisting of a mixture of anionic and nonionic surfactant agents, potassium hydroxide, and stabilizing agents (Cui et al., 2019).

1. Remove any adhesive labels from the bottles before washing.

2. Add approximately 10 mL of acetone to each bottle to be washed and close with a cap.

3. Shake the bottles vigorously one by one.

4. Discard the acetone.

5. Prepare a 2–5% Decon solution (or equivalent product e.g., 10% alkaline detergent TFD4) in hot water in a 20 L open container or sink.

6. Submerge the acetone-rinsed bottles and caps in the Decon solution overnight.

7. The next day, remove the bottles and caps from the solution, scrub every bottle and cap vigorously with the Decon solution using a cleaning brush, and rinse them thoroughly three times with tap water.

8. Submerge the bottles and caps in clean tap water in a container or sink for 24 hours.

9. Remove the bottles, rinse them with clean tap water and dry them for 6–8 hours upside down on a rack or dry the bottles and caps in an oven at 50°C for 20–30 minutes. Increase the drying time if moisture is still visible in the bottle.

Wash procedure in places where Decon or TFD4 is not available

1. Remove any adhesive labels from the bottles before washing. SOP for testing insecticide susceptibility of adult mosquitoes in WHO bottle bioassays.

2. Rinse the bottles with acetone, if available, as described above.

3. Prepare a 10% soap solution in hot water in a 20 L open container or sink.

4. Submerge the bottles and caps in the soapy water for 24 hours.

5. Remove them from the soapy water, and either i) scrub every bottle and cap vigorously with soap solution using a cleaning brush and rinse them thoroughly three times with tap water, or ii) wash them in a washing machine with hot water.

6. Submerge the bottles and caps in clean tap water in a container or sink for 24 hours.

7. Remove the bottles, rinse them with clean tap water and dry them for 6–8 hours upside down on a rack, or dry the bottles and caps in an oven at 50°C for 20–30 minutes. Increase the drying time if moisture or droplets are still visible in the bottles.

To ensure that no insecticide residues are left in the washed bottles, it is recommendable to check the wash quality by selecting some dry bottles at random and exposing 5 mosquitoes in each, recording their knockdown at the end of 1 hour of exposure and mortality at 24 hours of holding post-exposure.

Define Current Use Practices

Does everybody use the same SOP?

Papers using the WHO bottle bioassay method have referenced the SOP: (WHO, 2022).

Are there differences of interpretation of the method?

Interpretation of the method is quite clear. The methods state to keep the bottle vertical during testing, to use mosquitoes within the age range of 3-5 days, the number of mosquitoes per test are specified as well as expected rearing conditions and starving the mosquitoes for 2 hours prior to testing.

Are there results obtained largely consistent between studies?

Only two studies have reported using the WHO Bottle bioassay method. (Corbel et al., 2023) and (Govoetchan et al., 2023). The AI/mosquito strains used are not comparable.

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

I2I are reviewing the CDC bottle and WHO bottle bioassay.

Identify Potential Sources of Variation

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

The WHO bottle bioassay enables evaluation of insecticides that are not suitable for impregnation onto Whatman no.1 filter papers. However, bottles are self-prepared by the user with insecticides being coated by hand, which is a potential source of variation and less consistent in comparison to using insecticide-impregnated papers all acquired from a central source. Variability in the users' techniques may lead to variability in results.

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

The WHO bottle bioassay has been used for PPF testing in (Corbel et al., 2023). It was adapted to include assessing mosquito oviposition 7 days after exposure.

The SOP is adapted for use with chlorfenapyr where mortality testing occurs after 72 hours.

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

There are knowledge gaps in:

- Understanding the consistency of bottle coating, despite guidance being given on coating bottles there could be variability between users.
- Mosquito behaviour during exposure to the AI. Insecticide delivery relies on mosquitoes contacting the bottles treated surface. How mosquitoes interact with the surfaces has never been fully elucidated or quantified.

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

N/A

References

Brogdon, W., & Chan, A. (2010). *Guideline for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle Bioassay*. 1–28.

Corbel, V., Kont, M. D., Ahumada, M. L., Andréo, L., Bayili, B., Bayili, K., Brooke, B., Pinto Caballero, J. A., Lambert, B., Churcher, T. S., Duchon, S., Etang, J., Flores, A. E., Gunasekaran, K., Juntarajumnong, W., Kirby, M., Davies, R., Lees, R. S., Lenhart, A., … Yadav, R. S. (2023). A new WHO bottle bioassay method to assess the susceptibility of mosquito vectors to public health insecticides: results from a WHO-coordinated multi-centre study. *Parasites and Vectors*, *16*(1). https://doi.org/10.1186/s13071-022-05554-7

Govoetchan, R., Odjo, A., Todjinou, D., Small, G., Fongnikin, A., & Ngufor, C. (2023). Investigating discriminating concentrations for monitoring susceptibility to broflanilide and cross resistance to other insecticide classes in Anopheles gambiae sensu lato, using the new WHO bottle bioassay method. *PLoS ONE*, *18*(3 March). https://doi.org/10.1371/journal.pone.0276246

Owusu, H. F., & Muller, P. (2016). How important is the angle of tilt in the WHO cone bioassay? *Malar J*, *15*, 243. https://doi.org/10.1186/s12936-016-1303-9

WHO. (2022). Standard operating procedure for testing insecticide susceptibility of adult mosquitoes in WHO tube tests. *World Health Organization*, *January*, 17p.

World Health Organisation. (2021). *Determining discriminating concentrations of insecticides for monitoring resistance in mosquitoes*.

Innovation to Impact
Pembroke Place
Liverpool L3 5QA UK

contact@innovation2impact.org
+44 151 702 9308

BILL & MELINDA GATES *foundation*

LSTM
LIVERPOOL SCHOOL OF TROPICAL MEDICINE
Since 1898