I2I Landscaping exercise

WHO Tube bioassay

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Acronym List

AI	Active ingredient
DDT	Dichlorodiphenyltrichloroethane
MoA	Mode of action
PBO	Piperonyl butoxide
WHO	World Health Organisation

Summary

Aim and key questions addressed	- Used to evaluate the susceptibility of adult mosquito vectors to insecticides. - This bioassay is a direct response-ro-exposure test
Context	- Laboratory
Test item	- Insecticide-filter paper
Mosquito population	- Laboratory reared and wild populations
Number of mosquitoes per replicate	- 15-30
Endpoints measured	- 1-hour knockdown - 24-hour mortality
Exposure time	- 1-hour
Holding time	- See relevant protocol for active ingredient tested
Indicative of personal protection	- No
Suitable chemistries	- Insecticides that can impregnate filter paper
Appropriate controls	- Negative control: Carrier oil-impregnated papers
Relevant stage of production pipeline	- Mosquito characterisation

Characterisation of output	- Endpoints for pyrethroids are well defined, however, new activie ingredients may have different outcomes and will need to be defined
Accessibility	- Materials are relavtively easy to set up, however, access to pre-impregnated filterpapers can prove more difficult
Cost	- Low
Level of validation and characterisation of outputs	– Key courses of variation have been address, with a recent assement of the impact of mosquito number and age – Multi-centre studies have been performed
Outstanding questions, gaps and priorities	- Not appropriate for quantifying strength of resistance
Key references, related SOPs, guidelines and publications	- Praulins, G., McDermott, D. P., Spiers, A., & Lees, R. S. (2022). Reviewing the WHO Tube Bioassay Methodology: Accurate Method Reporting and Numbers of Mosquitoes Are Key to Producing Robust Results. Insects, 13(6). https://doi.org/10.3390/insects13060544 - WHO. (2022b). Standard operating procedure for testing insecticide susceptibility of adult mosquitoes in WHO tube tests. World Health Organization, (January), 17p

Overview (Praulins, McDermott, Spiers, & Lees, 2022)

Accurately monitoring insecticide resistance in target mosquito populations is important to combating malaria and other vector-borne diseases, and robust methods are key. The "WHO susceptibility bioassay" has been used for +60 years: mosquitoes of known physiological status are exposed to a discriminating concentration of insecticide. Several changes to the test procedures have been made historically which may seem minor but could impact bioassay results. The published test procedures and literature for this method were reviewed for methodological details. Areas where there was room for interpretation in the test procedures or where the test procedures were not being followed were assessed experimentally for impact on bioassay results: covering or uncovering of the tube end during exposure, number of mosquitoes per test unit, and mosquito age. Many publications do not cite the most recent test procedures, methodological details are reported which contradict the test procedures referenced or methodological details are not fully reported. As a result, the precise methodology is unclear. Experimental testing showed that using fewer than the recommended 15-30 mosquitoes per test unit significantly reduced mortality, covering the exposure tube had no effect, and using mosquitoes older than 2-5 days old increased mortality, particularly in the resistant strain. Recommendations are made for better reporting of experimental parameters.

Define Accepted Methodologies

Are there existing standard SOPs/Guidelines detailing methodologies?

The most recent WHO guidelines were updated in 2022 (WHO, 2022b), however there are multiple historical documents which outline previous versions of this methodology.

Are these sufficiently detailed?

Methodology is detailed however there are vagaries around some aspects of the methodology including:

- Number of mosquitoes per test unit

- The age of mosquitoes to be used for testing (this has changed over time with iterations of guidelines)
- Sample size (only recently defined)
- Temperature and humidity during testing (slight variations)

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

These methods require access to a WHO tube test kit and pre-treated insecticide papers. Little training is needed for conducting the assay, except for paper making training if not using pre-treated papers.

Are there issues with the methods or their interpretation?

It is often unclear if reported mortality is uncorrected or (Abbots) corrected. Often the raw numbers are not reported, and mortality may be depicted in graphical form, making it challenging to interpret results.

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

Many active ingredients (AI) have been tested. Commonly assessed AI include: DDT, dieldrin, malathion, fenthion, fenitrothion, OMS-33, propoxur, chlorphoxim, permethrin, deltamethrin, llambda-cyhalothrin, cyfluthrin, etofenprox, bendiocarb, carbosulfan, alpha-cypermethrin, pirimiphos-methyl, piperonyl-butoxide (PBO). The WHO recommended doses required for each AI vary depending on the AI and the target mosquito species (e.g., *Anopheles* or *Aedes*). Recommended doses have not been established for all AI/species.

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

The assay has been validated by historical use and multiple published studies using this method. These include multicentre studies (old and recent), which have been reviewed uder the I2I project:

- G. Praulins *et al.* (Praulins et al., 2022) Reviewing the WHO Tube Bioassay Methodology: Accurate Methods Reporting and Number of Mosquitoes Key to Producing Robust Results. https://www.mdpi.com/2075-4450/13/6/544

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

- The number of mosquitoes per test unit
- The orientation of the tube (horizontal or vertical) - The vertical orientation of the test tubes during performance of the bioassay was further justified in these test procedures, as horizontal positioning avoids the knockdown and recovery of mosquitoes, since knocked down mosquitoes would lie on treated paper instead of the untreated mesh-end of the test unit and so still be exposed to the insecticide. This would increase the exposure of the mosquito, and the exposure route may not be through the tarsi of the mosquito
- If the tube should be covered or uncovered to light - Based on three replicate tests, there was no evidence that covering the top of the exposure tubes with a cardboard disc during the exposure period had any impact on either 1 h knockdown or 24 h mortality. The rationale for the covering of the exposure tubes using in the test procedures is that it will prevent light entering through the mesh and so should discourage mosquitoes from resting on the upper mesh of the test units during exposure, which reduces their contact with the insecticide. It was not possible to assess if there was a reduction in resting on the mesh, as it was not possible to observe mosquito behavior during the exposure period, as the exposure chamber was covered by the insecticide-treated filter paper and the cardboard disc. However, due to the lack of significant difference in mortality seen in this study, we would suggest that this step appears to be unnecessary. So long as all test units are treated the same in terms of lighting, mosquitoes resting on the mesh should be consistent between test units and therefore there should have no impact on the final mortality scoring (Praulins et al., 2022).

- The age of mosquitoes to be used for testing (has changed over time with iterations of guidelines)
- The required sample size (only recently defined)
- Temperature and humidity (slight variations)

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

Endpoints standardly measured are 1 hour knockdown and 24 hour mortality. Additional endpoints may be required for new AIs with novel modes of action e.g., slow acting insecticides. In the currently WHO guidelines (WHO, 2022b) this only states "i.e. 24 hours post-exposure or *longer for slow-acting compounds*" and is not clearly specified per compound.

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

- SOP for making insecticide treated papers (WHO, 2022a)

Define Current Use Practices

Does everybody use the same SOP?

Multiple versions of the guidelines are available. Multiple SOP's across sites and institutions have been developed based on these guidelines, however the most recent iteration of the guidelines are not consistently referenced.

Are there differences of interpretation of the method?

Differences in interpretation defined above.

- number of mosquitoes per test unit
- age of mosquitoes to be used for testing (has changed over time with iterations of guidelines)
- required sample size (only recently defined)
- temp and humidity (slight variations)

Are there results obtained largely consistent between studies?

Results between studies are usually not comparable as mortality data for the same strains is usually for a susceptible strain and so mortality is 100% for these strains by this method.

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

Some optimisation and refinement underway as part of I2I review to identify potential sources of variation in the methodology.

Identify Potential Sources of Variation

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

- Number of mosquitoes per test unit - When varying the number of mosquitoes per test unit, mortality in this same experimental set up was unaffected by mosquito numbers between 15 and 30 mosquitoes per test unit. However, when only 10 mosquitoes were added per test unit, the 24 h mortality was significantly lower (7% compared to 50%). The same trend is not seen in the proportion of mosquitoes knocked down immediately post exposure, with knockdown being reduced in treatments with 15 and 20 mosquitoes per test unit compared to covered and uncovered treatments containing 25 or 30 mosquitoes. Knockdown thus appears to be positively correlated with the number of mosquitoes per test unit in this laboratory strain. This implies that mosquitoes are being differentially exposed during the bioassay, depending on the number of individuals within a single test unit (Praulins et al., 2022).
- Age of mosquitoes to be used for testing (has changed over time with iterations of guidelines) - When investigating the effect of mosquito age at the time of testing, we found that mosquitoes both 2 and 4 days older than the recommended testing age (2–5 days) show an increased susceptibility to permethrin. This increased susceptibility is seen at 6–9

days old for Kisumu and 4–7 days old for Tiassalé 13. This difference could be due to the increased fitness cost caused by resistance mechanisms in the Tiassalé 13 strain compared with the susceptible Kisumu strain (Praulins et al., 2022).

- Required sample size (only recently defined)
- Tempurature and humidity (slight variations)

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

The current endpoints may need redefining if this method is adapted for novel modes of action. This method is not currently used for chlorfenapyr, ivermectin or pyriproxyfen testing and is only validated for insecticides with mortality as the primary endpoint.

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

New methods may be required for alternative MoA's.

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

There are gaps in our understanding of the effect of different carrier oils on insecticide efficacy. The current method uses silicone oil for pyrethroids, however historically risella oil has been used for organochlorines and olive oil is used for carbamates and organophosphates. More work is needed to investigate how different carrier oils may impact the activity of new chemistries and to decide whether silicone oil will be the standard going forward for new AIs.

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

There are no current issues that need to be addressed with the methodology following the review. Noise bioassay data is being collected for this methodology and will further inform prioritization for this method going forward.

References

Praulins, G., McDermott, D. P., Spiers, A., & Lees, R. S. (2022). Reviewing the WHO Tube Bioassay Methodology: Accurate Method Reporting and Numbers of Mosquitoes Are Key to Producing Robust Results. *Insects*, *13*(6). https://doi.org/10.3390/insects13060544

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