I2I Landscaping report:

Tunnel test

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Acronym List

AI	Active ingredient
BFI	Blood-feeding inhibition
I-ACT	Ifakara Ambient Chamber Test
ITNs	Insecticide-treated nets
LITE	Liverpool Insect Testing Establishment
MoA	Mode of action
SOP	Standard operating procedures
WHO	World Health Organisation

Summary

Aim and key questions addressed	- Used as a secondary confirmatory assay when cone test results for a net sample are below a WHO defined threshold (i.e., <80% mortality or <95% knockdown) - Used to demonstrate that a net can deter blood-feeding against a rodent bait – intended to allow nets with excito-repellent properties to demonstrate they are effective
Context	- Laboratory
Test item	- Insecticide-treated nets (ITNs)
Mosquito population	- Laboratory reared
Number of mosquitoes per replicate	- 50
Endpoints measured	- Blood-feeding inhibition - 24-hour mortality (delayed mortality for newer active ingredients) - Fertility - Fecundity
Exposure time	- Overnight
Holding time	- See relevant protocol for active ingredient tested
Indicative of personal protection	- No
Suitable chemistries	- Chemistries applied to ITNs

Appropriate controls	- Negative control: untreated netting (made of polyethylene or polyester ideally equivalent fabric to test item) - Positive control: new, unused samples of relevant net product
Relevant stage of production pipeline	- Durability assessment
Characterisation of output	- The endpoints for the tunnel test are clearly defined as mortality,blood-feeding inhibition, fertility/fecundity but may need to be redefined if adapted for novel insecticide modes of action
Accessibility	- Not easily accessible - The need for an animal license, animal maintenance and ethical approval are major barriers to entry for many institutions
Cost	- More expensive than other laboratory assays due to specialised equipment required, mosquito number per replicate and animal costs
Level of validation and characterisation of outputs	– Little validation for newer active ingredients – The following variables are not well described: variation between animal bait, arrangement and size of holes in nets, mosquito number per assay, number of biological replicates per net piece
Outstanding questions, gaps and priorities	- Key weakness of this assay is the need to use an animal bait which is a barrier for some institutions and also raises questions on the use of non-human bait to assess behaviour of anthropophilic mosquitoes. - Validation is required across multiple testing sites
Key references, related SOPs, guidelines and publications	- World Health Organization. (2013). Guidelines for laboratory and field-testing of long-lasting insecticidal nets. - WHO Prequalification of Vector Control Products .Bioassay methods for insecticide-treated nets: tunnel test. (WHO, 2023)

Overview

The tunnel test is used to investigate the biological activity of a material's surface (untreated or treated with an active ingredient) under controlled laboratory conditions. The exposure of mosquitoes at a net interface in the wind tunnel is representative of interaction with the test material and observations are made on the relevant effects (e.g. mortality and blood feeding success) on mosquitoes within a host seeking experimental chamber. Nets washed at least 20 times that do not meet the criteria in the WHO cone test in laboratory studies should undergo tunnel tests.

The efficacy of treated nets may be underestimated if judges based on the outcome of standard cone bioassays. This is true particularly for insecticides that have a high excito-repellent effect, such as permethrin and etofenprox. In such cases, the efficacy (mortality and blood-feeding inhibition [BFI]) of nets washed 20 times or more than no longer meet the criteria in standard cone bioassays should be studies in a tunnel test.

The assay is carried out in a laboratory by releasing non-blood-fed, female anopheline mosquitoes aged 5–8 days into the tunnel made of glass/acrylic (see Figure 1). The tunnel comprises three chambers 1) netted release chamber (25 x 25 x 25cm), 2) response chamber (60 x 25 x 25cm with a volume of 37,500cm3) and 3) netted collection chamber (25 x 25 x 25cm). The treated net sample to be tested is attached to a 25 x 25cm frame (e.g. cardboard) and slotted into the WHO tunnel. 50 mosquitoes are introduced into the cage and are free to fly. The mosquitoes must pass through a holed netting sample to reach an animal bait (e.g. guinea pig or rabbit) for mosquito biting. After taking a blood meal, the mosquitoes may fly back to the cage at the end of this compartment and rest. A tunnel with untreated netting is always used as a negative control.

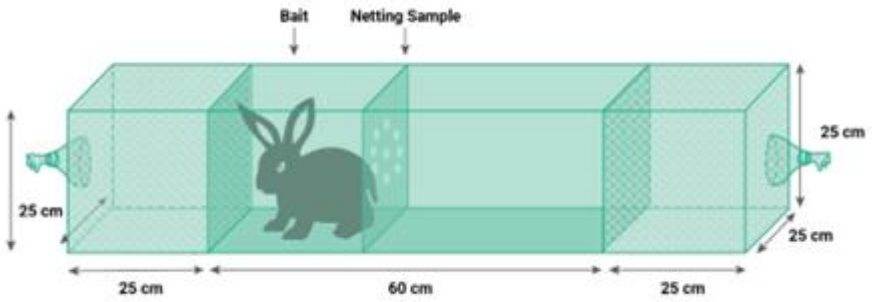

Figure 1: Tunnel test

Define Accepted Methodologies

Are there existing standard SOPs/Guidelines detailing methodologies?

- Guidelines for laboratory and field testing of long-lasting insecticidal nets (WHO., 2005).
- Guidelines for laboratory and field-testing of long-lasting insecticidal nets (WHO., 2013)
- Guidelines for monitoring the durability of long-lasting insecticidal mosquito nets under operational conditions (WHO., 2011).
- WHO Prequalification of Vector Control Products .Bioassay methods for insecticide-treated nets: tunnel test. (WHO, 2023)

Are these sufficiently detailed?

Methodology is detailed however there are vagaries around certain parameters within the published literature including:

- Age of mosquitoes
- Number of mosquitoes exposed per replicate
- Testing conditions (temperature and humidity)

The most recent WHO, 2024 guidelines are sufficiently detailed, providing information which includes the background, equipment, parameters on the mosquito's required for testing and environmental conditions, endpoints of the assay and information on data analysis.

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

These methods require access to WHO tunnel test equipment and training is required for the setup, operation and cleaning of the equipment. The need for a live animal with this method requires an animal handling license and specific training. Laboratories need dedicated space for animal rearing, animal maintenance and the ability to run multiple tunnel tests simultaneously.

Are there issues with the methods or their interpretation?

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

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

Many pyrethroid only nets and 'next generation' nets have been tested using this method.

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

The tunnel test is not validated but has been used extensively in published studies across multiple sites.

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

- Mosquito strain – characterisation of mosquito strains before use in test (Lees et al., 2022)
- Number of mosquitoes / mosquito density per replicate
- Animal bait / blood source
- Environmental conditions e.g. temperature and humidity during testing and where the mosquitoes are held for delayed mortality monitoring
- Performing control and treated nets simultaneously
- Exposure length
- Mosquito age
- Time at which tests were conducted
- Post exposure holding times

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

The endpoints are clearly defined in the WHO, 2023 guidelines. These are as follows:

- Mortality at 24 hours (and delayed mortality if required)
- Blood feeding inhibition
- Fertility – Eggs per female
- Fecundity-proportion of fertile females

Mosquitoes are collected in each section of the tunnel test apparatus and then split into different categories: alive and blood-fed, dead and blood-fed, alive and non-blood-fed, dead and non-blood-fed. Mosquitoes are kept for further endpoints e.g. fertility/fecunditiy.

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

- Animal handling protocols which should follow each institutions guidelines

- I2I-SOP-006 -Tunnel Test
- LITSOP015-Mosquito rearing for colony maintenance and testing

Define Current Use Practices

Does everybody use the same SOP?

Multiple versions of the guidelines are available, however various parameters are reported differently across published studies (mosquito number, length of exposure time).

Are there differences of interpretation of the method?

Differences in interpretation of the method are explained in more detail throughout this report.

- Number of mosquitoes used per test replicate
- Age of mosquitoes to be used in testing
- Environmental conditions – temperature and humidity
- Length of exposure time
- Source of blood meal

Are there results obtained largely consistent between studies?

This comparison between studies has not been possible due to differences in testing conditions/mosquito/insecticide combination. The endpoints mortality and blood-feeding inhibition has often been presented in studies in graphical form making interpretation of results difficult.

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

- The animal bait used in this methodology are non-preferred hosts for malaria mosquitoes, especially the highly anthropophagic vectors *Anopheles gambiae*, *funestus* and *arabiensis*.
- The Ifakara Ambient Chamber Test (I-ACT) (Massue et al., 2019) has been proposed an alternative method that could be used instead of the WHO tunnel test (see 'I2I Landscaping Exercise: Ifakara Ambient Chamber Test').

Identify Potential Sources of Variation

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

- Experimental conditions (temperature and humidity) need to be monitored and recorded throughout the testing exposure time.
- Number of replicates performed each night needs to be recorded, along with whether controls were performed at the same time.
- Number of mosquitoes used per test replicate.
- Care of animal bait- e.g. any treatment with insecticides for flea/worm control or diet with pesticides could effect the results. Animal care should be documented and kept consistent between studies.
- Net samples-storage conditions -storage conditions prior to testing should be documented and batch numbers reported
- If the treated net sample is attached to a frame that is reusable it must be a material that can be cleaned to remove and pesticide residues. Insect rearing to monitor mosquito fitness, for example, average mosquito weight and wing length, are a requirement to

ensure consistent results. Mosquito fitness data should be presented in study reports and standardised methods of mosquito rearing used.

- The time of conduct of tests should be consistent-the upregulation of enzymes occurs at the start of the dark phase of mosquito circadian rhythm which can strongly impact results.

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 (MoA). Currently the WHO tunnel test is the main laboratory assay for testing chlorfenapyr as other bioassays with a shorter exposure time, and less room for 'free flying' activity are not considered suitable.

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

- It is costly and raises welfare concerns to use animals as live bait.
- Animal baits are not the preferred host for the main vectors of concern. Is there a potential to different baits in these assays? An experiment is currently underway in the Liverpool Insect Testing Establishment (LITE) to investigate the possibility of using non-animal baits in the tunnel test.
- The cost of using 50 mosquitos per test replicate can become expensive.
- Length of exposure time – does current overnight exposure exaggerate the length of time a mosquito would realistically contact a net?

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

An experiment is currently underway in the LITE to investigate the possibility of synthetic attractants to replace a live animal bait.

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

The tunnel test needs to be validated, especially for use with next generation nets, across multiple sites.

References

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