I2I Landscaping exercise

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	- 100
Endpoints measured	- Blood-feeding inhibition - 24-hour mortality (delayed mortality for newer active ingredients)
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 (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 and blood-feeding inhibition, 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 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 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.

Overview

The tunnel test is used to measure the mortality and blood-feeding success of host-seeking mosquitoes in an 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 tunnel test assay is performed in a laboratory by releasing non-blood-fed female mosquitoes aged 5-8 days old into a 60cm tunnel (25cm x 25cm square section) made of glass (Figure 1). At each end of the tunnel a 25cm square cage covered with polyester netting is fitted. The treated net sample to be tested is held in a disposable cardboard frame and placed at one third the length of the glass tunnel. The surface area available to the mosquitoes is 400cm^2 (20cm x 20cm), with nine holes each 1cm in diameter: one hole is located at the center of the square, and the other eight are equidistant and located 5cm from the border. In the shorter section of the tunnel, a suitable animal bait (e.g., guinea pig or rabbit) is places, which is unable to move and is available for mosquito biting. One hundred female mosquitoes are introduced into the cage at the end of the longer section of the tunnel. They are free to fly in the tunnel but have to make contact with the piece of netting and locate the holes in it before passing through to reach the bait. 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.

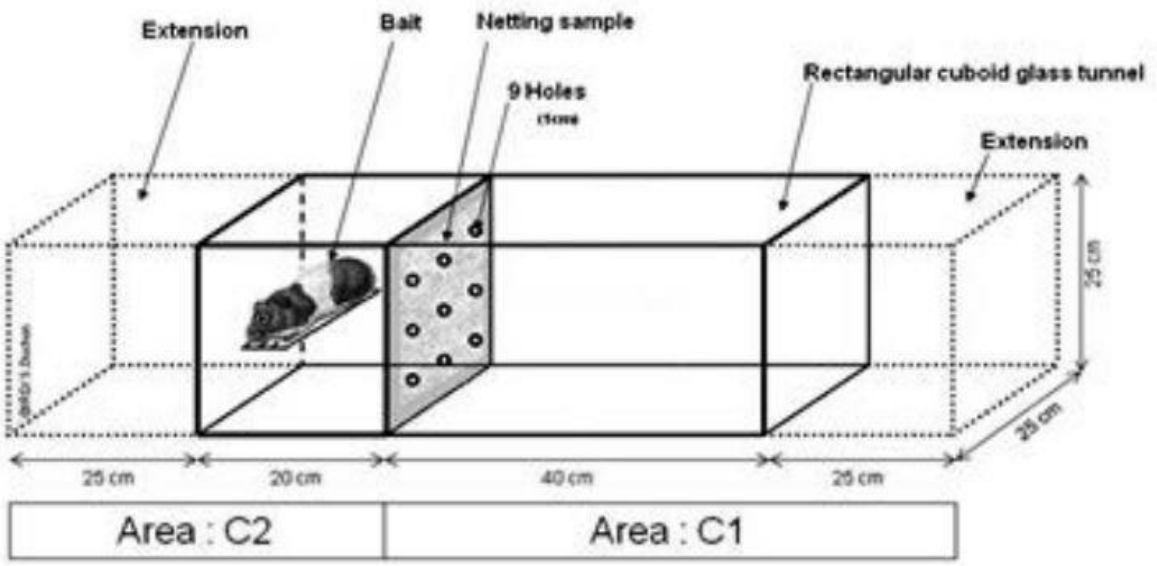

Figure 1. Tunnel used for studying the efficacy of insecticide-treated nets.

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).

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)

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

These methods require access to WHO tunnel test 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
- Testing conditions – temperature and humidity
- Performing control and treated nets simultaneously
- Exposure length
- Mosquito age

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

The endpoints for the tunnel test are defined as mortality and blood-feeding inhibition in the WHO guidelines. 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.

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

- Animal handling protocols which should follow each institutions guidelines
- Mosquito rearing SOPs

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?

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*.
- A study by Kamande (Kamande *et al.*, 2022) investigated the use of different blood sources and altering mosquito density. Results suggest that the WHO tunnel test using a rabbit bait may be ran with 50 mosquitoes instead of 100, which would increase the sample sizes needed for bio-efficacy durability monitoring. Using a membrane feeder

with 50 mosquitoes is a potential replacement if control blood feeding rates can be improved

- 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.

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 100 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

Kamande, D. S., Odufuwa, O. G., Mbuba, E., Hofer, L., & Moore, S. J. (2022). Modified World Health Organization (WHO) Tunnel Test for Higher Throughput Evaluation of Insecticide-Treated Nets (ITNs) Considering the Effect of Alternative Hosts, Exposure Time, and Mosquito Density. *Insects*, *13*(7). https://doi.org/10.3390/insects13070562

Lees, R. S., Armistead, J. S., Azizi, S., Constant, E., Fornadel, C., Gimnig, J. E., … Spiers, A. (2022). Strain Characterisation for Measuring Bioefficacy of ITNs Treated with Two Active Ingredients (Dual-AI ITNs): Developing a Robust Protocol by Building Consensus. *Insects*, *13*(5). https://doi.org/10.3390/insects13050434

Massue, D. J., Lorenz, L. M., Moore, J. D., Ntabaliba, W. S., Ackerman, S., Mboma, Z. M., … Moore, S. J. (2019). Comparing the new Ifakara Ambient Chamber Test with WHO cone and tunnel tests for bioefficacy and non-inferiority testing of insecticide-treated nets. *Malaria Journal*, *18*(1), 1–15. https://doi.org/10.1186/s12936-019-2741-y

World Health Organization. (2005). Guidelines for laboratory and field-testing of long-lasting insecticidal nets. *Who/Htm/Ntd/Whopes/2013.1*, 93. Retrieved from www.who.int/about/licensing/copyright_form/en/index.html

World Health Organization. (2011). *Guidelines for monitoring durability of long-lasting insecticidal nets under operational conditions*. *2*(3), 136–137. https://doi.org/10.1007/s101980100075

World Health Organization. (2013). *Guidelines for laboratory and field-testing of long-lasting insecticidal nets*.

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