I2I Landscaping report:

Video Cone Test

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Key contributors: Annabel Murphy-Fegan, Katherine Gleave, Jeff Jones

Acronym List

AI	Active Ingredient
An.	Anopheles
BORIS	Behavioural Observation Research Interactive Software
ITN	Insecticide-treated net
LITE	Liverpool Insect Testing Establishment
LSTM	Liverpool School of Tropical Medicine
µg/ml	Microgram per milliliter
Mm	Millimetre
PPF	Pyriproxyfen
SD	Standard deviation
SOP	Standard operating procedure
WHO	World Health Organisation
ViCTA	Video Cone Test Analysis

Summary

Aim and key questions addressed	- Used as a method to video record the standard cone test in the presence of a human host. This enables extra behavioural parameters to be detected and measured in addition to mortality which is useful for product evaluation and comparing product performance between different mosquito populations.
Context	- Laboratory
Test item	- Mosquito
Mosquito population	- Laboratory reared or field collected
Number of mosquitoes per replicate	- 5
Endpoints measured	- Knockdown, mortality, behavioural measurements e.g. net contact, total movement, regional movement
Exposure time	- 3 minutes
Holding time	- 24 hours. Longer if sub-lethal work done, dependent on study
Indicative of personal protection	- N/A
Suitable chemistries	- Insecticide-treated net (ITN), any treatment chemistry
Appropriate controls	- Untreated netting, Permanent 2.0

Relevant stage of production pipeline	- Product development; has potential for use in evaluating ITNS following a period of use
Characterisation of output	- Endpoints well defined. Adaptation required for active ingredients with a different mode of action. Additional sub-lethal work dependent on study
Accessibility	- Materials and set up are easily accessible. Need to be sourced and training is required
Cost	- Specialised equipment is required; the setup is similar to the standard WHO cone test but has equipment modifications. Specialised training is required to carry out analysis on the recorded videos
Level of validation and characterisation of outputs	- Multi-site study has been conducted for validation purposes; data analysis in underway
Outstanding questions, gaps and priorities	- N/A
Key references, related SOPs, guidelines and publications	- Hughes, A. et al. (2022) 'A closer look at the WHO cone bioassay: video analysis of the hidden effects of a human host on mosquito behaviour and insecticide contact', Malaria Journal, 21(1), p. 208. Available at: https://doi.org/10.1186/s12936-022-04232-4 - Jones, J. et al. (2023) 'Video augmentation of the WHO cone assay to quantify mosquito behavioural responses to insecticide-treated nets', Parasites & Vectors, 16(1), p. 420. Available at: https://doi.org/10.1186/s13071-023-06029-z - Cone Bioassay- I2I-SOP-004

Overview

The World Health Organisation (WHO) cone test is the current standard method to evaluate mosquito mortality induced by direct contact with a treated surface and is the most widely used assay for evaluating the efficacy of insecticidal netting. During testing, adult female mosquitoes are exposed for 3 minutes under a clear plastic cone, on a substrate angled at 45 degrees as shown in Figure 1.

Figure 1: Set up of a standard WHO cone test board. Image from LITSOP007.

The WHO cone test commonly measures two outputs: knockdown at 60 minutes and mortality at 24 hours, though active ingredients with different modes of action may require mortality measurement beyond 24 hours. Despite knockdown and mortality being important parameters, there are additional factors which are also useful for assessing a product and mosquito resistance, e.g. levels of excito-repellency or contact irritancy and overall contact time with a net. The video cone test assay was developed to allow characterization of behavioural responses to ITNs through the incorporation of a host attractant, enabling a more realistic assessment of mosquito behaviour at the net interface and the addition of video capture during the 3min test, indicative of the mosquito’s response to that a.i. and suitable for detailed analysis. Due to the ongoing challenges of insecticide-resistant vectors the development of existing tools such as

ITNs with new technology is essential. The video cone test is useful for providing in-depth information on new active ingredients on nets.

The set up is similar to the standard WHO cone test and is shown in Figure 2 with the addition of a host attractant.

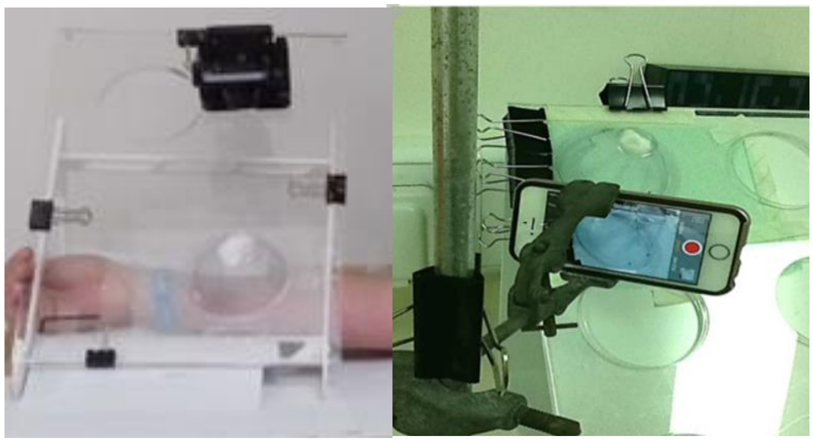

Figure 2: Video cone apparatus. A) cone board with camera phone mounted using a phone mount attached to the cone board (ViCTA board) B) standard WHO cone board with camera mount using a clamp stand. Image from LITSOP007.

There are two alternative methods available for analysis, dependent upon the outputs required for the specific study. The videos can be analysed using scan sampling where data on mosquito positions: (whether in flight, cone contact or net contact) are recorded at 5 second intervals. There is also semi-automated analysis using a software designed within LSTM called ViCTA (Video Cone Test Analysis by Jeff Jones) which captures the activity of each mosquito during testing, an example is shown in Figure 3. ViCTA assesses at 0.1s intervals, 1800 timepoints over a 3-minute assay. The data can then be analysed to obtain:

- Total mosquito activity: movements of all five mosquitoes in the cone over 180 seconds (s) enabling behaviours to be compared between mosquito strains and ITN types.
- Regional activity: movements of all five mosquitoes in the cone are stratified into the upper half and lower half of the cone volume and represented as mean proportion activity. Information from this parameter indicates irritancy/repellency from the bed net.

- Evolution of activity over time: measurement of lag time (s) between exposure and detectable behavioural response which is useful for determining contact irritancy and repellency.

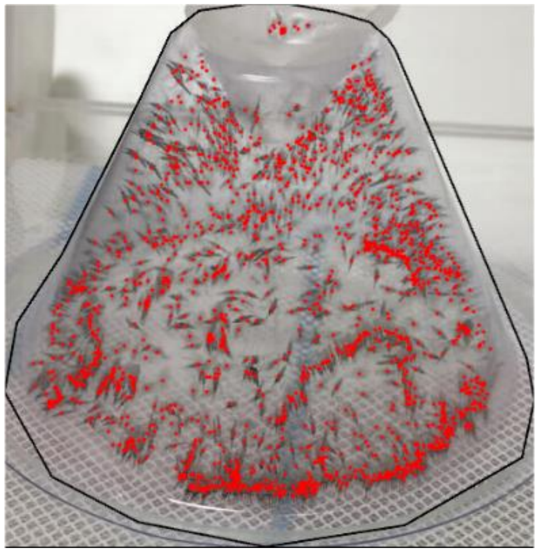

Figure 3: Detected mosquito movement by the ViCTA system during the WHO Cone Assay. Image from https://essentials.lstmed.ac.uk/our-approach/video-cone-tests, date accessed 29/01/24.

These behaviours can then be linked to endpoints such as longevity and ability to blood feed post exposure.

Define Accepted Methodologies

Are there existing standard SOPs/Guidelines detailing methodologies?

An SOP has been developed by the I2I:

- Cone Bioassay- I2I-SOP-004
- Hughes *et al.*, (2022) gives detailed steps of the procedures carried out.

Are these sufficiently detailed?

The SOP contains a sufficient level of detail and includes thorough step-by-step guidelines on performing assay and instructions on performing the analysis.

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

Training is required for the performance of a cone test assay, including setting up the cone boards, aspiration of mosquitoes and the use of a vacuum pump, though this is a standard mosquito bioassay.
Additional specialised equipment is required to conduct the video cone test, the setup of a smartphone to record mosquito activity and a cushioned arm rest to position the forearm at a fixed distance of 5mm from the netting. These adaptations are shown in Figure 2.

Specialised training is required for the free and open access analysis software BORIS (Behavioural Observation Research Interactive Software) available from the University of Turin. This software records mosquito behaviour using scan sampling and the Video Cone Test System (ViCTA) software to detect mosquito movement.

Are there issues with the methods or their interpretation?

This method is less high throughput than the standard WHO cone test method. The operator must be present throughout the 3-minute assay, using their arm as bait during the experiment, meaning only a single cone can be tested at a time by an operator. In comparison, the standard WHO cone test, shown in Figure 1 enables multiple cones to be tested simultaneously, by staggering time intervals of mosquitoes aspirated on and off the cone at e.g. 1-minute intervals between testing. The scan sampling method of analysis, if required, post assay also adds additional time although the ViCTA analysis is more rapid.

For the equipment, there are two alternative set ups which are suggested for recording the video assay, shown in Figure 2. The cone test board shown in Figure 2 A is set up with the cone positioning and smart phone fixed in place on a pre-made stand. The set up shown in Figure 2 B uses a moveable phone stand. Despite the SOP advising that the net and cone are positioned so that the cone is in full focus, using the fixed set up is more consistent and standardised between replicates. It does, however, add additional costs due to the production of the custom-made, specialised board.

The assay is carried out in the light to enable visibility for video recording. If the assay was carried out in darkness this could potentially increase host seeking behaviour as *Anopheles* host seek at night. However, this is designed to be a simple bench top assay and recording in the dark would require a more complex engineering set up.

The scan sampling method also requires data to be collected on mosquito positions at 5 second intervals. Behaviours occurring between these time points are missed, and scan sampling at different time points e.g. at 3 or 10 seconds, could produce different results. Scan sampling also does not enable the operator to determine whether time spent in contact with the net is a result of resting or host seeking.

Additionally, there is small blind spot in filming the bioassay due to the shape of the cone (this can be seen in Figure 3). This could result in data being lost since a mosquito may not be visible during parts of the film, affecting analysis. To minimise this the amount of parafilm used to block the entrance hole should be minimal and should always be used instead of cotton wool. This issue has more of an impact in studies counting the number of mosquito points to confirm total detected movements, as opposed to tests analysing changes in distribution of activity from lower to upper regions.

During filming, the tapering shape of the cone causes the sides as they are seen from the camera to be slightly darker and more blurred, these sides can partially distort the view. The positional viewpoint of a 2D camera also affects the perception of movement. The angle of the camera in the video cone test is chosen so that see patterns of movement over the entire net surface can be seen, however at this angle bias can occur if a mosquito is at the bottom of the cone resting on the net and crawls slowly across the net towards the top of the camera region would falsely interpret that the mosquito is moving upwards. Alternatively, if the camera is directly in line with the cone so that the bottom of the net is not visible a mosquito walking along the net would not have its movement detected. Crawling on nets is mostly observed on untreated nets used as the control, flight behaviour is more commonly observed on treated nets where this issue with the angle of the lens would not be an issue. With flight behaviour also, the shape of the cone is problematic. The space available to fly is less at the top of the cone than the bottom and therefore the mosquito's 'choice' of movement is affected by the tapering inner walls of the cone which restricts its movement. This could be overcome by using a square box instead of the cone, however the assay would no longer be based on the widely used and known standard WHO test.

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

Anopheles gambiae sensu lato (insecticide susceptible: Kisumu and N'gousso and resistant: Banfora and VK7) have been tested in published data available, with and without a host attractant on untreated, PermaNet 2.0 and Olyset nets in Hughes *et al.*, (2022). Jones *et al.*, (2023) tested the same mosquito strains and nets, with the addition of PermaNet 3.0.

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

There are two published studies Hughes *et al.*, (2022) and Jones *et al.*, (2023) which measured different outputs and treatments. Further work is ongoing to complete validation. Parameters that have been tested in the published studies are net contact, cone contact, total and regional mosquito in the cone, resting behaviour, knock down/mortality, blood feeding, blood meal size and mosquito longevity.

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

The equipment required is well detailed in the SOP. There are two options available for the smartphone set up. Both ensure that the net and cone are in focus, the use of the same set up, however, would improve standardisation between tests.

The SOP details that the bioassay requires 5 non-blood-fed adult female mosquitoes aged 2-5 days and the use of two controls. The removal of 10% sucrose solution, replaced with water only is approximately 20 hours prior to testing (late afternoon the day before) and if required remove the water 4 hours before testing begins. 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 video cone procedure SOP, with knock down being measured 1 hour after exposure and the mortality again 24 hours later. Adaptations are required however depending on the type of active ingredient used where mode of action may differ e.g. testing with chlorfenapyr, a pro-insecticide where mortality measurement beyond 24 hours is required.

In addition to knock down and mortality, Hughes *et al.*, (2022) and Jones *et al.*, (2023) also quantified post-exposure blood feeding, and ingested blood meal size. This provides more representative estimates of ITN efficacy by assessing the mosquito's ability and success of blood feeding post insecticide exposure.

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

- LITSOP123- Test preparation, detailing the set-up of equipment required including holding cups.
- LITSOP142-Equipment Cleaning in the LITE Laboratory Area, detailing general cleaning after testing.

- LITSOP007-WHO cone bioassay. The video cone bioassay SOP details performing the bioassay as per the WHO cone bioassay, with the exceptions detailed.
- SOP I2I-SOP-004-SOP from I2I on standard cone testing.

For cleaning procedures refer to 'LITSOP007- WHO cone bioassay' and 'LISOP142 Equipment cleaning in the LITE laboratory area.'

Define Current Use Practices

Does everybody use the same SOP?

The video cone test assay is sufficiently standardised and has been used within research and for product development. In the published data Hughes *et al.,* (2022) has thoroughly detailed the methods used, and Jones *et al.,* (2023) reported using the same method. There are some differences between these and the 'Video Cone Procedure' LITSOP159. These are as follows:

- The testing age in the SOP is specified as 2-5 days, Hughes *et al.,* (2022) tests at 3-5 days.
- The starvation time prior to testing of 10% sugar solution was 20 hours in the SOP and 5 hours in Hughes *et al.*, (2022).
- 2 behavioural parameters were measured in Hughes *et al.*, (2022): 'In contact with the net' or 'in flight'. The SOP also recommends: 'resting on the cone.'
- Hughes *et al.,* (2022) and Jones *et al.,* (2023) also measured post exposure blood feeding, ingested blood meal size and longevity. These additional parameters are not detailed in the SOP.

Are there differences of interpretation of the method?

Interpretation of methods from the SOP is clear. The experimental set up is detailed, the age of testing mosquitoes, the acclimatisation period prior to testing and the distance of the host arm from the net.

The number of mosquitoes per cone are defined but not the quantity of mosquitoes required per mosquito/insecticide combination, this will be dependent upon the specific study protocol.

Are the results obtained largely consistent between studies?

As different behavioural parameters were analysed between the two studies which have been performed to date this aspect cannot be compared. Hughes *et al.,* (2022) measured contact with the net and flight behaviour whereas Jones *et al.,* (2023) assessed total mosquito and regional mosquito activity.

Both studies measured blood feeding and longevity, however. In Hughes *et al.,* (2022) mortality in susceptible strains meant that blood feeding was only measured on resistant strains, in comparison Jones *et al.,* (2023) found that some Kisumu and N'gousso lived long enough to feed after exposure to Interceptor G2.

Both studies obtained similar results for the mean blood meal size in Banfora and VK7 2014 on untreated nets at: 12.67 µg/ml (SD=6.65) and 13.25 µg/ml (SD=6.39), in Hughes *et al.,* (2022) and 10.48 ug (SD=6.42) for Banfora and 13.10 ug (SD: 8.93) for VK7 2014 in Jones *et al.*, (2023). Jones *et al.*, (2023) also reported that after ITN exposure blood meal weights decreased in N'gousso and VK7 2014 strains, which is consistent with Hughes *et al.,* (2022) who found that meal sizes were consistently smaller after ITN exposure.

Hughes *et al.*, (2022) found that median longevity with untreated netting was 14 days for each strain, (bar VK7 2014) which is consistent with Jones *et al.*, (2023) who found that the median longevity was 12-14 days.

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

This method is sufficiently standardised to be used to collect data on nets for product development and durability monitoring, although it has not been routinely used to date for these purposes. Work is ongoing for a formal validation process of this method.

Identify Potential Sources of Variation

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

The nets used for testing are all obtained from the manufacturer, however variations in surface insecticide content between net pieces cut even from the same net is a potential source of variability in testing.

Results may differ based on different operators/hosts performing the assay due to inter-person variation. Hughes *et al*., (2022) used the same host for all assays to remove this source of variability, although this is not specified in the published SOP and may be logistically difficult. Jones *et al*., (2023) used the same methods as Hughes *et al*., (2022) specifying a host attractant was used. The presence of other hosts in the room or in close proximity could also potentially alter behaviour of mosquitoes within the cone which is not mentioned in the SOP.

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

The SOP states to measure knock down 1 hour after exposure and mortality at 24 hours. Adaptations are required however depending on the type of AI used where mode of action may differ. For example, chlorfenapyr, a pro-insecticide measuring mortality beyond 24 hours is required. Some active ingredients also require additional end points to be measured such as egg laying for pyriproxfen (PPF), an insect growth regulator which causes female sterilisation.

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

The video cone test quantifies information on knockdown and mortality as well as behavioural characteristics, enabling the comparison of behaviours such as irritancy or repellency between mosquito strains and bed net types giving a more in-depth interpretation on how mosquitoes interact with insecticides. The additional sub-lethal work done in Hughes *et al.*, (2022) and Jones *et al.*, (2023) on longevity, blood feeding and blood meal size also enables useful information to be obtained beyond short-term responses.

References

Hughes, A. *et al.* (2022) 'A closer look at the WHO cone bioassay: video analysis of the hidden effects of a human host on mosquito behaviour and insecticide contact', *Malaria Journal*, 21(1), p. 208. Available at: https://doi.org/10.1186/s12936-022-04232-4.

Jones, J. *et al.* (2023) 'Video augmentation of the WHO cone assay to quantify mosquito behavioural responses to insecticide-treated nets', *Parasites & Vectors*, 16(1), p. 420. Available at: https://doi.org/10.1186/s13071-023-06029-z.

INNOVATION TO IMPACT

Innovation to Impact
Pembroke Place
Liverpool L3 5QA UK

contact@innovation2impact.org
+44 151 702 9308

BILL & MELINDA GATES foundation

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