I2I Landscaping report:

WHO cone bioassay for ITNs

Last updated: July 2024

Key contributors: Giorgio Praulins, Katherine Gleave, Annabel Murphy-Fegan

Acronym List

AI	Active ingredient
ITN	Insecticide-treated net
LLIN	Long-lasting insecticidal net
MoA	Mode of action
WHO	World Health Organisation

Summary

Aim and key questions addressed	- Used to assess bioefficacy of insecticide-treated nets (ITNs) and treated surfaces against a well characterised mosquito strain by forcing mosquitoes into close proximity with active ingredients - ITN regeneration time, wash-resistance, and efficacy studies - Comparing nets sampled from the field at timepoints after distribution can detect longitudinal changes in bioefficacy
Context	- Laboratory
Test item	- Insecticide-treated nets (ITNs)
Mosquito population	- Laboratory reared
Number of mosquitoes per replicate	- 5
Endpoints measured	- 1-hour knockdown - 24-hour mortality (and delayed mortality) - Fecundity - Fertility
Exposure time	- 3-minutes
Holding time	- See relevant protocol for active ingredient tested
Indicative of personal protection	- No
Suitable chemistries	- Chemistries applied to ITNs and treated surfaces - Not suitable for nets with excito-repellent properties
Appropriate controls	- Negative control: untreated netting (ideally equivalent fabric to test item)

	- Positive control: new, unused examples of relevant ITN product or treated surface
Relevant stage of production pipeline	- Product development - Bioefficacy assessment - Durability assessment
Characterisation of output	- Endpoints for pyrethroid nets and pyrethroid synergist nets are well defined. Endpoints have been defined for ITNs with different active ingredients such as chlorfenapyr and pyriproxyfen, however these are new additions to the guidelines and so need to be validated
Accessibility	- Materials and set-up are easily accessible
Cost	- Low
Level of validation and characterisation of outputs	– Validated with a wealth of historical data, however, current endpoints may need redefining if this method is adapted for novel modes of action.
Outstanding questions, gaps and priorities	- More work is needed to correlate the bioavailability of insecticides with regards to mosquito bioassays and chemical net surface analysis
Key references, related SOPs, guidelines and publications	- Lees, R. S., Armistead, J. S., Azizi, S., Constant, E., Diabaté, A., Fornadel, C., … Oxborough, R. (2022). Strain Characterisation for Measuring Bioefficacy of ITNs Treated with Two Active Ingredients (Dual-AI ITNs): Developing a Robust Protocol by Building Consensus. https://doi.org/10.20944/preprints202203.0345.v1 - World Health Organization. (2013). Guidelines for laboratory and field-testing of long-lasting insecticidal nets. - World Health Organization. (2023). WHO Prequalification of Vector Control Products. Bioassay methods for insecticide-treated nets: Cone test.

Overview

The World Health Organization (WHO) cone bioassay plays an integral role in the evaluation of the efficacy of long-lasting insecticidal nets (LLINs) as well as insecticides used in indoor residual spraying. This bioassay investigates the biological activity of a material's surface under standardised laboratory conditions, with observations made on the effects on mosquitoes, including knock down (KD) and mortality. The test is used on materials that can be treated or untreated with an active ingredient (AI). This SOP details the process for conducting a cone bioassay in laboratory LLIN testing.

For a standard WHO cone bioassay (World Health Organization, 2023) precisely five susceptible, non-blood-fed, 3–5-day-old female Anopheles (species to be stated in the test report) mosquitoes are exposed to each piece of insecticide-treated netting (25 cm x 25 cm or 30 x 30cm) for 3 minutes under standard WHO cones. Dependent upon the study protocol, the mosquitoes are then held for a set period of time, either 24 hours or an extended time period (e.g. 72 hours is required for fertility measurements) with access to 10% sugar solution. Knock-down is recorded 60 minutes after exposure and mortality at 24 hour time periods until the end of holding time~~s~~. Additional sub-lethal measurements also recorded include: 1) Fertility (quantity of eggs laid per female) and 2) fecundity (the proportion of fertile females).
One piece each from four different nets, should be tested. Up to four cones at a time may be attached to a piece of netting, and five mosquitoes at one time should be exposed in a cone at an angle of 45-60° (angle kept consistent within a study), This procedure should be repeated until a total of 50 mosquitoes have been exposed to each piece. Results should be reported for each net tested and for the four nets (4 pieces x 10 cone tests x 5 mosquitoes = 200 mosquitoes). Mosquitoes exposed to untreated net pieces are used as controls; they should be tested each day, just before and just after testing treated netting material. If the mortality in controls on any day is < 10%, the results for that day should be adjusted by Abbott's formula. If the mortality in controls is > 10% at 24 hours and 20% after extended holding periods beyond 24 hours,, the results for that day are considered invalid and should be discarded. Bioassays should be carried out at 27 ± 2 °C and 85% ± 20% relative humidity.

The definitions of mortality and knock-down are those recommended by the WHO (World Health Organization, 2023). Mosquitoes are considered to be alive if they can both stand upright and fly in a coordinated manner. Mosquitoes that are moribund or dead are classified and recorded as knocked down at 60 minutes and as dead at 24 hours. A mosquito is moribund if it cannot stand (e.g., has one or two legs), cannot fly in a coordinated manner or takes off briefly but falls immediately. A mosquito is dead if it is immobile, cannot stand or shows no signs of life.

The cone test was established to assess the bioefficacy of insecticide treated nets and surfaces. Guidelines exist for its use in laboratory (historically termed 'Phase I') studies (net regeneration, wash-resistance, and efficacy studies), and community ('Phase III') trials (durability of bioefficacy studies). In some instances, this method has been adapted to measure mosquito behaviour at the net interface, however this is not yet validated.

Accepted Methodologies

Are there existing standard SOPs/Guidelines detailing methodologies?

There are several iterations of the guidelines:

- W.H.O., Test procedures for insecticide resistance monitoring in malaria vectors, bio-efficacy and persistence of insecticides on treated surfaces: report of the WHO informal consultation, Geneva, 28-30 September 1998 (World Health Organization, 1998),
- W.H.O., Guidelines for laboratory and field testing of long-lasting insecticidal nets 2005 (World Health Organization, 2005),
- W.H.O., Guidelines for testing mosquito adulticides for indoor residual spraying and treatment of mosquito nets 2006 (World Health Organization, 2006),

- W.H.O., Guidelines for Monitoring the Durability of Long-Lasting Insecticidal Nets 2011 (World Health Organization, 2011),
- W.H.O., Guidelines for laboratory and field testing of long-lasting insecticidal nets 2013 (World Health Organization, 2013).
- W.H,O., Prequalification of Vector Control Products. Bioassay methods for insecticide-treated nets: Cone test. (World Health Organization. 2023).

Are these sufficiently detailed?

The current methodology is sufficiently detailed. Multiple groups have investigated the effect of different iterations on certain parameters specified in the method.

A review on the minimum number of mosquitoes to use to get an accurate result with cone tests was performed using LLINs from Madagascar. Results reported that the minimum number of mosquitoes that need to be tested is at least 40 mosquitoes, which is an improvement on the WHO recommended number of 100 mosquitoes. (Boyer *et al.,* 2018).

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

These methods require access to a WHO cone, cone board and tilted stand. Little training is needed for conducting the assay.

Are there issues with the methods or their interpretation?

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

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

The WHO cone test has been used to assess a range of pyrethroid nets and dual active ingredient net products containing an additional insecticide, or synergist. Pre-qualified vector control ITNs which the method can be used for are listed in Table 1.

Table 1. Product name and active ingredients of pre-qualified vector control insecticide-treated nets.

ITN Product Name	Active Ingredient / Synergist
DuraNet LN	Alpha-cypermethrin
DuraNet Plus	Alpha-cypermethrin, Piperonyl Butoxide (PBO)
Interceptor	Alpha-cypermethrin
Interceptor G2	Alpha-cypermethrin, Chlorfenapyr
MAGNet	Alpha-cypermethrin
NiraNet	Alpha-cypermethrin
OLYSET Net	Permethrin
OLYSET PLUS	Permethrin, Piperonyl Butoxide (PBO)
Panda Net 2.0	Deltamethrin
PermaNet 2.0	Deltamethrin
PermaNet 3.0	Deltamethrin, Piperonyl Butoxide (PBO)
Reliefnet Reverte	Deltamethrin
Royal Guard	Alpha-cypermethrin, Pyriproxyfen
Royal Sentry	Alpha-cypermethrin
Royal Sentry 2.0	Alpha-cypermethrin
SafeNet	Alpha-cypermethrin
Tsara	Deltamethrin
Tsara Boost	Deltamethrin, Piperonyl Butoxide (PBO)
Tsara Plus	Deltamethrin, Piperonyl Butoxide (PBO)
Tsara Soft	Deltamethrin
VEERALIN	Alpha-cypermethrin, Piperonyl Butoxide (PBO)
Yahe LN	Deltamethrin
Yorkool LN	Deltamethrin

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 across multiple sites. There is a wealth of publicly available data in published literature and from the WHO.

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

- The number of mosquitoes per test unit
- The insecticide resistance status of mosquito strain if testing dual-AI nets (Lees et al., 2022)
- The age of mosquitoes to be used for testing (has changed over time with iterations of guidelines)
- Environmental conditions during testing and post-exposure holding (temperature and humidity)
- Insecticide-treated net storage conditions
- Time of testing (should align with the mosquitoes circadian rhythm)

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

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

- Knockdown at 60 minutes
- Mortality at 24 hours (and delayed mortality if required)
- Fertility – Eggs per female (standard post holding time 72 hours)
- Fecundity-proportion of fertile females

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

- SOP: Net washing for laboratory LLIN trials (I2I-SOP-006) (https://innovationtoimpact.org/wp-content/uploads/2022/07/Net-washing-for-laboratory-LLIN-trials_Updated-May-2022.pdf)
- WHO Guidelines for laboratory and field testing of long-lasting insecticidal nets (World Health Organization, 2013)
- Washing procedure from the Collaborative International pesticides Analytical Council (CIPAC)
- I2I-SOP-004 -Cone Bioassay
- LITSOP015-Mosquito rearing for colony maintenance and testing
- – 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.
- 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.

Current Use Practices

Does everybody use the same SOP?

A review of published literature and compiled comment from those working in industry, has shown that people generally use the most recent iteration of the WHO cone test, however, the methodology has remained unchanged and so choice of guidelines for referencing should not impact the way that the bioassay is performed.

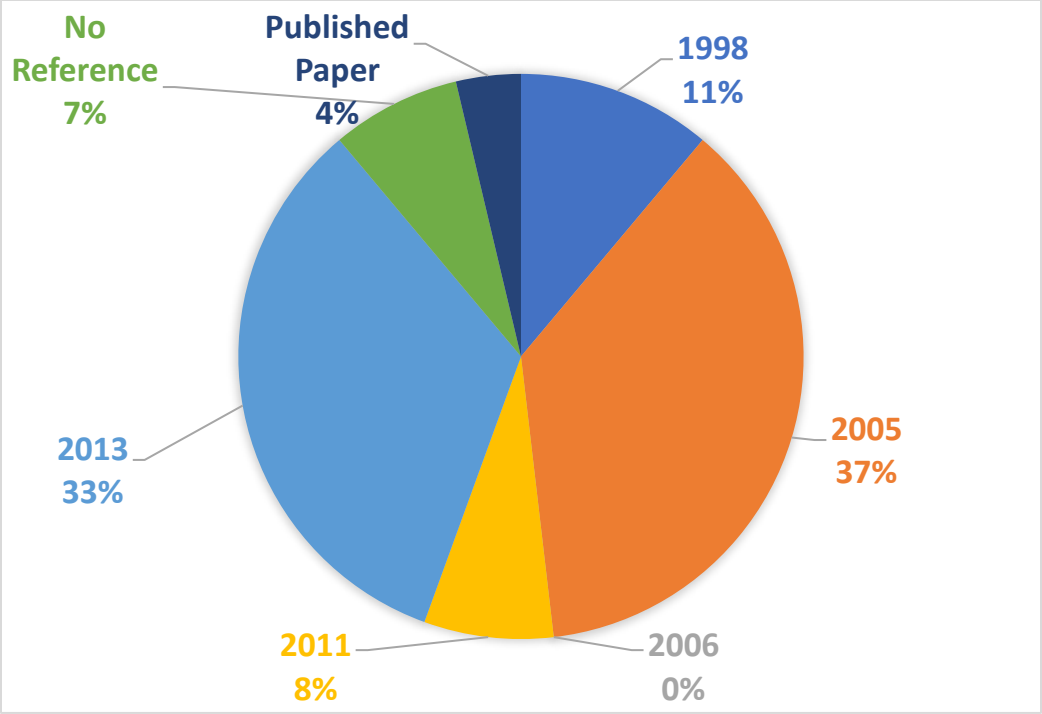

Figure 1. Referenced WHO guidelines for performing a WHO cone test.

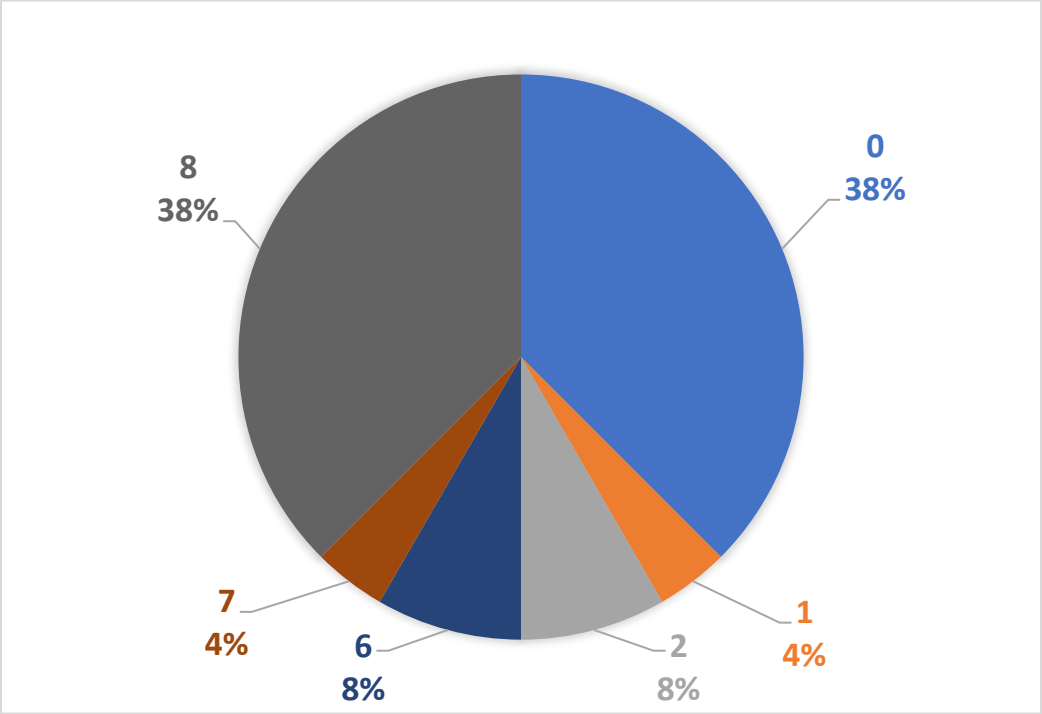

Figure 2. The number of years out of the date the referenced protocol used was in published literature.

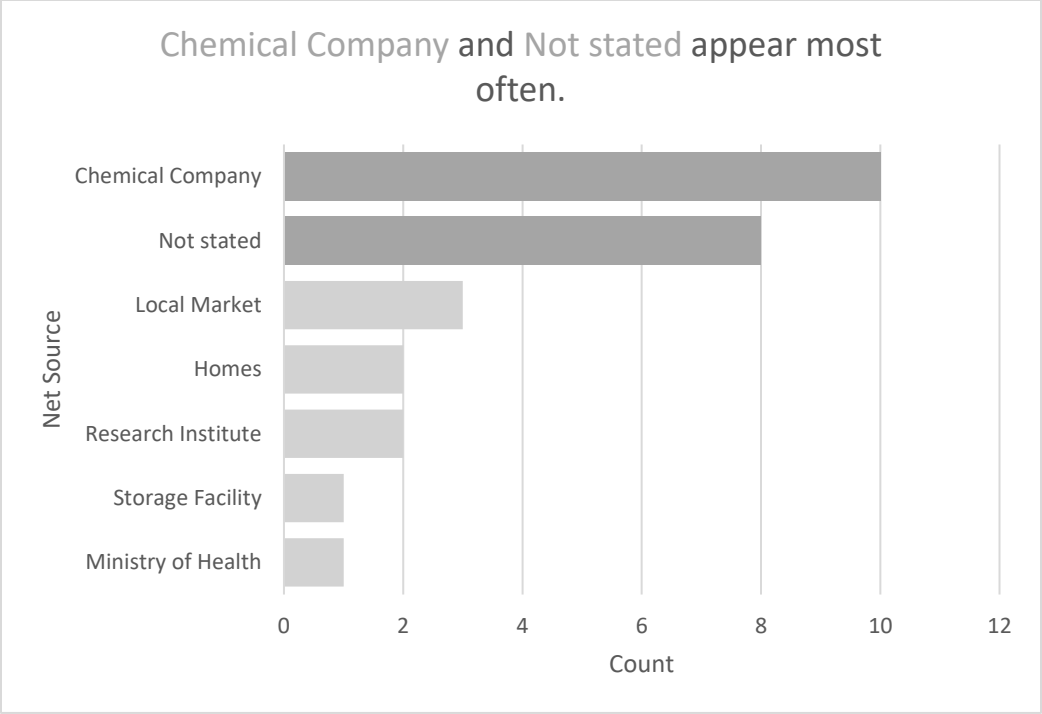

Figure 3 – Reported sound of insecticide-treated net used in WHO cone test.

Table 2. Storage conditions of insecticide-treated nets used in WHO cone test.

Conditions	Count
Not stated	13
Ambient Temperature	5
Foil and fridge	2
Foil	1
Plastic bag and ambient temperature	1
Plastic bag	1
30 °C and 75–85% RH	1
Stored in envelope	1
Foil and plastic bag	1
30°C and dark	1

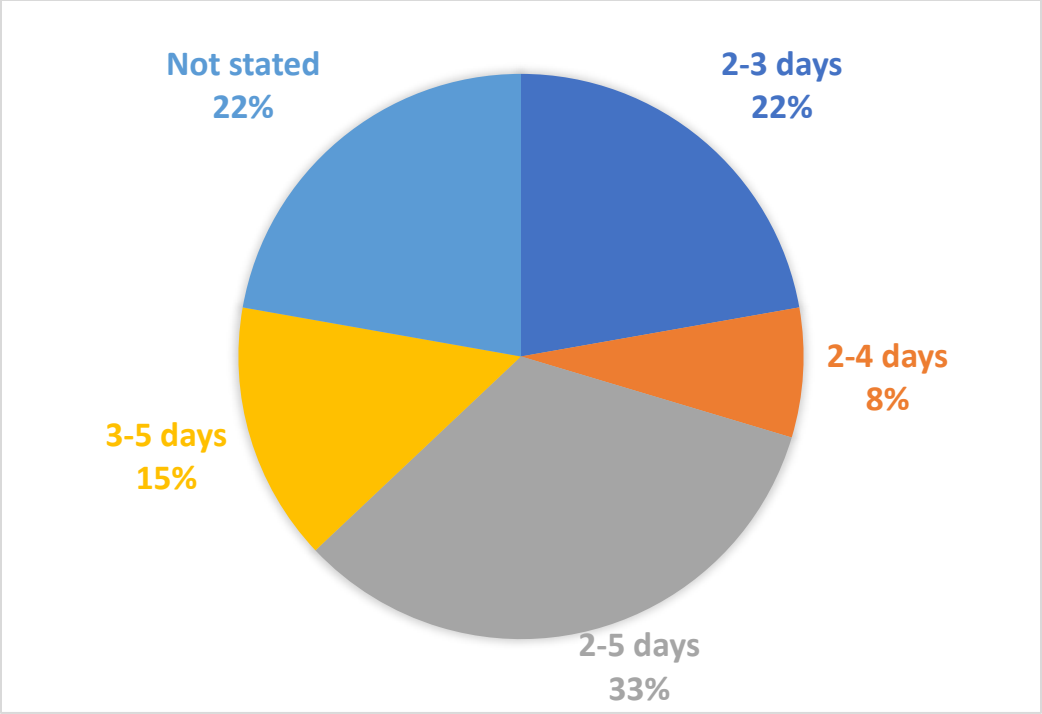

Figure 4 - Age of mosquitoes used in WHO cone test.

Table 3. Net swatch size used in WHO cone test.

Size	Count
25 x 25cm	9
30 x 30cm	7
Whole net	6
Not stated	5

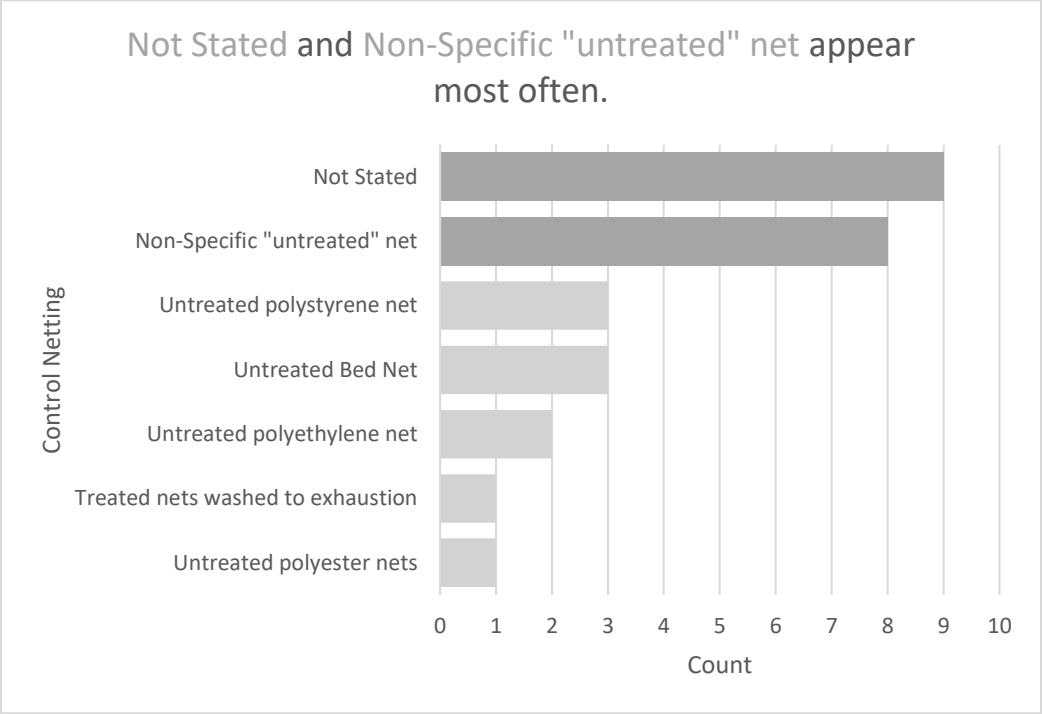

Figure 5 - Negative control netting used in WHO cone test.

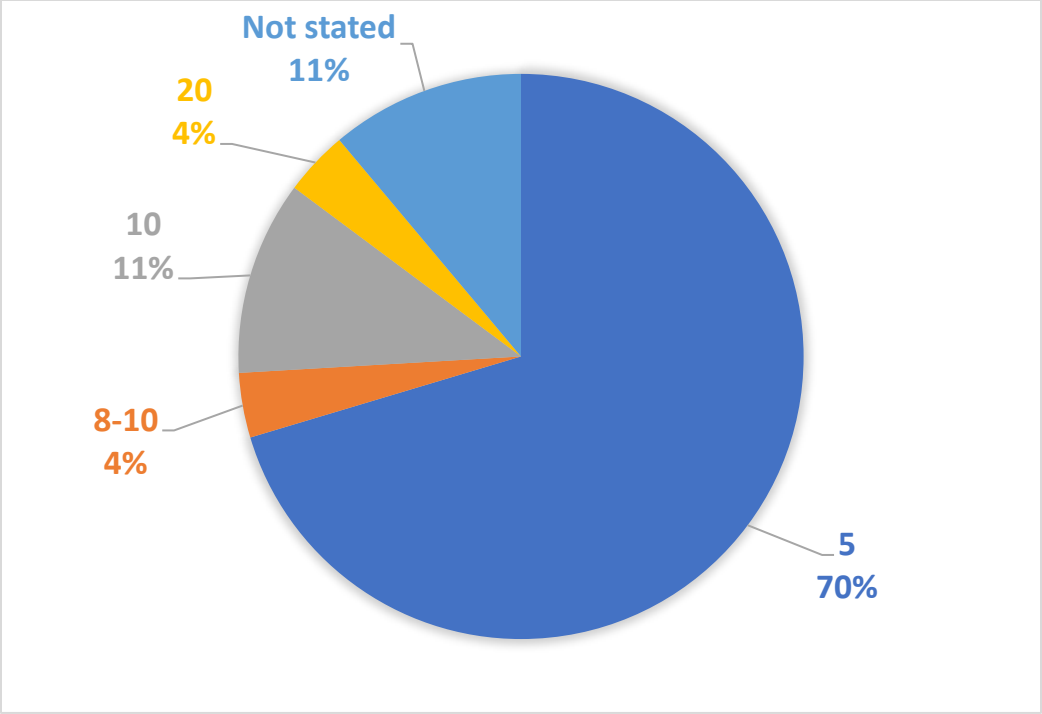

Figure 6 – Mosquitoes per cone

Additional parameters of the WHO cone test that differ between use in published literature:

- Samples taken per net varied between 1 – 10 subsamples
- Number of nets used varied between 1 – 932 nets
- Mosquito sample size varied between 20 – 20000 mosquitoes
- Number of cones used per subsample varied between 1 – 16 cones

Are there differences of interpretation of the method?

Despite 5 mosquitoes per cone being stated in the guidelines, several published studies used between 8 and 10 mosquitoes per cone. Bar this, the method appears to be largely interpretated and carried out in the same way across users.

Are there results obtained largely consistent between studies?

Susceptible strains often show results that largely agree with each other, however these are often mortalities of 100%, or close to 100%, so it can prove difficult to know how consistent results between studies are.

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

There is some optimisation and refinement underway as part of I2I to identify potential sources of variation in the methodology.

Potential Sources of Variation

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

Sources of variability in the method include:

- Number of mosquitoes per test unit
- Age of mosquitoes used for testing
- Temperature and humidity
- Operator handling
- Required mosquito sample size
- Required number of cone tests per net
- Required number of net pieces to be tested
- Configuration of the bioassay board

- Variations in surface insecticide content between net pieces cut even from the same net is a potential source of variability in testing. Net samples-storage conditions -storage conditions prior to testing should be documented and batch numbers reported
- 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.

Noise bioassays, similar to that performed by I2I for the WHO tube and bottle tests, could help to investigate sources of variability.

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

The current endpoints include measuring delayed mortality and fecundity/fertility measurements which is useful for chemistries with modes of action that require measurement beyond mortality at 24 hours.

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

New methods may be required for alternative MoAs.

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

There are gaps in our understanding of the bioavailability of insecticides on the net surface. More work is needed to correlate the bioavailability of insecticides with regards to mosquito bioassays and chemical net surface analysis. I2I are currently undertaking a piece of work around this topic.

The cone test is not an effective method for investigating the potential entomological efficacy of an ITN. The cone test alone can also not be used to quantify surface concentrations of AI, it can only indicate the presence of insecticide on the surface by measurement of the mosquito endpoints following exposure.

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

The number of mosquitoes used per cone needs to be addressed. Hughes (PhD thesis) investigated this:

- Results from bioassays using five individuals were compared to bioassays using ten individuals. For each strain and treatment, there was no significant different in knock-down and 24-hour mortality between five and ten test individuals.
- Susceptible mosquito strain and resistant mosquito strain knock-down and mortality data obtained in the WHO standard cone test using five individuals was used to compare with ten individuals. These assays were carried out at different time points and with different mosquito batches so results must be viewed with caution.
- After exposure to PermaNet, five and ten individuals of both susceptible strains showed consistent knock-down and mortality of between 98% (+2 SEM) - 100%. After exposure to Olyset, with both five and ten individuals of Kisumu, knock-down did not exceed 58% (±4.9 SEM) and mortality 10% (±4.5 SEM). Similarly, for N'gousso, knock-down did not exceed 20% (±6.3 SEM) and mortality 12% (+8 SEM). For the two resistant strains, although knock-down increased in Tiassalé against PermaNet, neither PermaNet nor Olyset induced mortality greater than 10% (+4.5 SEM). Five individuals were used in each replicate in subsequent experiments in line with WHO recommendations.

Previous work has also been undertaken to investigate the angle of the cone board and found that mosquitoes (both pyrethroid resistant and pyrethroid susceptible strains) spent more time on the net at a 60-degree angle than at the WHO guideline recommended 45 degrees (Owusu

& Muller, 2016). The current guidelines suggest to test between 45-60 degrees and to keep this consistent within studies to reduce variability.

The configuration of the bioassay board was investigated by (Koinari et al., 2022) in 'WHO cone bioassay boards with or without holes: relevance for bioassay outcomes in long-lasting insecticidal net studies'.

- Conducted study to investigate whether circular holes in the bioassay board intended to 'force the mosquitoes to stand on the net surface' lead to systematic bias in the key bioassay endpoints (60-minute knockdown and 24-hour mortality).
- Study performed at two sites and results not only varied with bioassay board configuration but also with mosquito colony. WHO cone bioassay results were systematically biased between the two facilities such that the use of *Anopheles* in one site predicted higher knockdown and mortality results.

References

Boyer, S., Pothin, E., Randriamaherijaona, S., Rogier, C., & Kesteman, T. (2018). Testing bio-efficacy of insecticide-treated nets with feer mosquitoes for enhanced malaria control. *Scientific Reports*, *8*(1), 1–8. https://doi.org/10.1038/s41598-018-34979-3

Koinari, M., Bubun, N., Amos, B., Kiari, K., Lahu, D., & Karl, S. (2022). WHO cone bioassay boards with or without holes: relevance for bioassay outcomes in long-lasting insecticidal net studies. *Malaria Journal*, *21*(1), 1–9. https://doi.org/10.1186/s12936-022-04412-2

Lees, R. S., Armistead, J. S., Azizi, S., Constant, E., Diabaté, A., Fornadel, C., … Oxborough, R. (2022). *Strain Characterisation for Measuring Bioefficacy of ITNs Treated with Two Active Ingredients (Dual-AI ITNs): Develop- ing a Robust Protocol by Building Consensus*. (March). https://doi.org/10.20944/preprints202203.0345.v1

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

World Health Organization. (1998). Test procedures for insecticide resistance monitoring in malaria vectors, bioefficacy and persistance of insecticides on treated surfaces. *Report of the WHO Informal Consultation, Geneva*, pp. 36–41. https://doi.org/10.1680/gftsltob.27374.0007

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. (2006). *Guidelines for testing mosquito adulticides for indoor residual spraying and treatment of mosquito nets*.

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*.

World Health Organization. (2023). *WHO Prequalification of Vector Control Products. Bioassay methods for insecticide-treated nets: Cone test*.

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