ANNEX 3. GUIDELINES FOR TESTING NEW LONG-LASTING INSECTICIDAL NET PRODUCTS TO SUBSTANTIATE EFFICACY CLAIMS IN AREAS OF HIGH INSECTICIDE RESISTANCE

Background

New long-lasting insecticidal nets (LLINs) are being developed by several manufacturers and advocated for use in areas where mosquito vectors are resistant to pyrethroid insecticides. In February 2014, the paradigm “vector control interventions for use in areas of high pyrethroid resistance” was assessed by the WHO Vector Control Advisory Group (VCAG). The paradigm was defined as a novel intervention or an adaptation of an existing product class that has an overall effect on vectorial capacity and reduces human infection or disease in areas where the local vectors have substantive pyrethroid resistance. Under this broad paradigm heading, VCAG has reviewed the data for two insecticide combination/mixture LLINs, and made a recommendation on the public health value of the paradigm of combination/mixture nets designed to have increased effectiveness in areas of high pyrethroid resistance.

The current document outlines the evidence that VCAG would expect to see to substantiate manufacturers’ claims of increased efficacy of combination/mixture LLINs compared with pyrethroid-only LLINs in areas of high (RR > 10-fold) insecticide resistance.1 VCAG will convene a specialist subgroup to evaluate and refine manufacturers’ claims of efficacy for their products against highly pyrethroid-resistant vector populations. This process is intended to supplement the current WHOPES evaluation procedures for classical LLINs. Further, all combination/mixture LLINs submitted to VCAG with claims of increased effectiveness in areas of high pyrethroid resistance should be well advanced in WHOPES efficacy and safety evaluations and in specification development.

Scope

This document addresses LLINs that are designed to have greater efficacy in areas of high insecticide resistance.2 Currently, most of these products would address resistance to pyrethroid insecticides and consist of combination/mixture nets, including pyrethroids plus another AI and/or synergist.

Objectives

As next-generation LLINs are likely to be more expensive than current LLINs, control programmes and donors will need information on whether these new nets are more effective at killing (or protecting against) insecticide-resistant populations. Current WHOPES guidelines do not require new LLINs to demonstrate superiority to in-use LLINs. Furthermore, the existing guidelines recommend that all initial testing of LLIN efficacy be performed on insecticide-susceptible mosquito populations, and new nets must demonstrate equivalency to conventional LLINs against susceptible mosquitoes, while recognizing such populations are increasingly difficult to find and resistance populations still generate useful data. In reality, new nets, particularly those not containing pyrethroids, may not perform as well

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1 This threshold (RR >10-fold) has been set to exclude mosquito strains with kdr-only based resistance mechanisms.
2 For guidance, at least a 25% improvement should be achieved and the comparator reference strain must be well documented. Manufacturers should specify the percentage improvement with confidence intervals (CIs), where the CIs are based on standard errors that reflect the variation between replicates.
as conventional LLINs against susceptible mosquitoes in WHOPES tests, but may greatly outperform conventional LLINs when resistant mosquitoes are used. New nets are urgently needed to help control pyrethroid-resistant mosquito populations, but it is clear that the current testing guidelines will not generate the data needed to adequately evaluate the performance of these products against these mosquito populations, and further specifications for net evaluation need to be agreed upon.

This document aims to provide guidelines for the minimum data that need to be generated in order to assess whether next-generation LLINs are superior to current LLINs in areas of high resistance. The following assumptions are made:

1. Next-generation LLINs are primarily designed to provide enhanced protection (compared with existing pyrethroid-only LLINs pre-/post-washing) against malaria transmitted by highly pyrethroid-resistant mosquitoes. Hence, all tests should be performed on well-characterized pyrethroid-resistant mosquito populations. It is important to realize that the resistance ratio is pertinent to protection and should be determined.

2. Based on previous studies on fully susceptible pyrethroid vectors, one can assume that if personal protection against highly pyrethroid-resistant mosquitoes is observed there will be protection against malaria in such settings.

3. Next-generation LLINs are evaluated on their ability to provide enhanced protection or increased mosquito mortality in areas of high pyrethroid resistance and not on their utility as a resistance management tool.¹

Note: Recommendations from this VCAG subgroup on LLIN efficacy against insecticide-resistant populations will relate to only the specific situations tested, and will not be generally applicable to all conditions of insecticide resistance. Nets will need to be appropriately matched to their target area based on the resistance ratio and detailed characterization of resistance profiles of local mosquitoes prior to in-country and regional use.

¹ Resistance management is a process, and evaluating the utility of individual products in this process will require a burden of evidence that is beyond the scope of this document.
EVALUATING LLIN EFFICACY AGAINST PYRETHROID-RESISTANT MOSQUITOES

Data generation will take a three-stage approach to reduce costs and allow the process to be stopped at each stage if increased efficacy is not apparent. These guidelines are intended to provide a general framework for evaluating next-generation LLINs. Detailed SOPs to follow will be available through the VCAG website.

1. STAGE I – LABORATORY TESTING

1.1. Objective
To demonstrate that the next-generation LLIN is significantly better at killing, reducing the reproductive capacity of and/or protecting against pyrethroid resistant mosquitoes compared to a pyrethroid-only LLIN.

1.2. What is meant by “significantly better”?  
   i. Next-generation LLINs should be compared to a standard WHOPES-recommended pyrethroid LLIN.1
   ii. All laboratory testing must be performed on at least three characterized industry standard pyrethroid-resistant mosquito strains (Appendix 1) or comply with the documentation requirements listed in Section 1.3.
   iii. Next-generation LLINs must demonstrate:
       − where insect mortality is the expected outcome, at least 25% increase in mortality compared with pyrethroid-only LLINs, following five replicates for both net types.
       − where insect mortality is NOT the expected outcome, at least 25% impact on the longevity, blood-feeding and/or reproductive output of the mosquitoes exposed to the new LLIN vs pyrethroid-only LLINs, with statistical significance.
   iv. Finally, improvements over current LLINs must be maintained after the requisite number of standardized washes.2

It is noted that percentage improvement in Phase 1 cone tests has limited operational significance due to poor correlation (or lack of calibration) with field results; however, for guidance, at least a 25% improvement should be achieved using a well-documented reference strain. Manufacturers should specify claims for percentage improvement with confidence intervals (CIs), where the CIs are based on standard errors that reflect the variation between replicate tests.

1.3. What resistance strains should be tested?  
   i. Standard strains that represent the broad spectrum of major insecticide resistance mechanisms currently known to exist in mosquito vector populations should act as the reference test strains for next-generation LLINs. A list of the standard strains of insecticide-resistant mosquitoes which may be procured for testing is given at the end of this document.

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2 The standard mosquito strains listed in Appendix 1 provide uniform comparators for all studies. Any alternative resistant strains used outside of those listed in Appendix 1 must comply with the documentation requirements described below.
ii. At least three strains must be tested, two of which must have major metabolic resistance mechanisms.

iii. Alternative strains: if alternative strains are used for assessment, the resistance mechanisms must be fully characterized at the time of testing. The results of the resistance profile and evidence demonstrating underlying resistance mechanisms should be documented within the dossier. The resistance level of any strain used for testing must be greater than 10-fold that of a susceptible strain of the same species at the LC50. During all testing, a laboratory-susceptible strain must also be run in parallel as a control.

1.4. What method should be used?
Robust demonstration of specific beneficial entomological end-points such as increased mosquito mortality prevention of blood-feeding or reduction of reproductive output is required. This should be demonstrated by:

• Cone bioassay undertaken as specified in WHOPES guidelines.
  − Exposure should be 3 min, with knockdown recorded at 60 min and mortality at 24 h.
  − If an AI has a documented mode of action that does not result in rapid knockdown and kill (e.g. a slow-acting insecticide), the time period for evaluating mortality may be extended; however, a rationale for the testing procedures used must be provided.
  − LLINs that do not demonstrate improvements in the cone tests should be tested by tunnel bioassays (see below), which will evaluate slow-acting or mechanistically alternative compounds.1

• Tunnel bioassay undertaken as specified in WHOPES guidelines.
  − Tunnel assays should be used if an AI functions by repellency (requiring testing on free flying insects), or if an AI requires an exposure of greater than 3 min to give operationally representative data in cone assays.
  − Tunnel tests should use the same strains of resistant mosquitoes as the cone bioassays.2

• For products that have a growth regulator AIs, measurements of reproductive output (oviposition, fecundity and fertility inhibition) will be needed.

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1 Tunnel bioassays test may reveal AI toxicity which is not apparent in cone or daytime contact bioassay, as mosquitoes are exposed to the treated nets at night, mimicking natural circadian host-seeking behaviours.

2 In some cases, resistant mosquito strains used for testing may not meet the 50% minimum blood feeding criteria for controls specified in the WHOPES guidelines. Alternative criteria can be considered on a case by case basis, however, a rationale for the testing procedures used must be provided.
Replicates for cone test

Cone tests should use standardized 2–5-day-old non-blood fed adult females only. The acceptable minimum number of replicates for each mosquito strain is as follows:

<table>
<thead>
<tr>
<th>Strain</th>
<th>Replicates</th>
<th>Total mosquitoes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1 (untreated net)</td>
<td>1 net x 10 replicates x 5 mosquitoes = 50 mosquitoes</td>
<td></td>
</tr>
<tr>
<td>Control 2 (pyrethroid-only LLIN)</td>
<td>4 nets x 10 replicates x 5 mosquitoes = 200 mosquitoes</td>
<td></td>
</tr>
<tr>
<td>Test nets</td>
<td>4 nets x 10 replicates x 5 mosquitoes = 200 mosquitoes</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>450 females per strain per new LLIN to be assessed</td>
</tr>
</tbody>
</table>

- A minimum of one laboratory-susceptible strain and three pyrethroid-resistant strains must be tested.
- Sample size calculations should be made in advance of any experimental work and sample size should be sufficient to demonstrate the minimum effect at 5% significance levels.
- Results should be discarded if mortality on the untreated net exceeds > 10%.

Replicates for tunnel test

Tunnel tests should use standardized 5-8 day old non-blood fed adult females only. The acceptable minimum number of replicates for each mosquito strain is as follows:

<table>
<thead>
<tr>
<th>Strain</th>
<th>Replicates</th>
<th>Total mosquitoes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1 (Untreated net)</td>
<td>3 replicates x 50 mosquitoes</td>
<td></td>
</tr>
<tr>
<td>Control 2 (Pyrethroid only LLIN)</td>
<td>3 replicates x 50 mosquitoes</td>
<td></td>
</tr>
<tr>
<td>Test nets</td>
<td>3 replicates x 50 mosquitoes</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>450 females per strain per new LLIN to be assessed</td>
</tr>
</tbody>
</table>

- Samples size calculations should be made in advance of any experimental work to clarify the size of effect expected and the minimum effect that can be detected.
- Results should be discarded if mortality on the untreated net exceeds 10%.

Note on mosaic and combination nets: In the case of nets where the sides and top of the net are not treated in an identical manner (e.g., differing in insecticide content and/or polymer type), data with 4 nets x 10 replicates x 5 mosquitoes for each surface type need to be generated. If the proposed mechanism of action is based on the mosquito contacting an insecticide and a synergist located on different parts of the net, accommodation should be made in the guidelines/SOPs for sequential exposure of mosquitoes to the two components; however, this must not assume that all mosquitoes will contact both parts of the net and therefore Phase II evaluation is essential to determine efficacy in field conditions.
1.5. Product quality assurance

Before laboratory, hut or community trials are undertaken, basic quality assurance should be in place to ensure that the products tested meet specifications for quality control (manufacturers or WHO, if available).

Manufacturers should provide a certificate when supplying the product for testing that states that the product meets WHO or manufacturer’s specifications for quality control. Quality assurance of the nets by high-performance liquid chromatography or gas chromatography should also be undertaken before the products are tested. Independent physical and chemical analyses of the products for compliance with specifications in an accredited, qualified laboratory may be required before efficacy testing.

All net testing should be undertaken on LLINs that have been washed once and left for the WHOPES-recommended regeneration time (or the time specified by the companies against insecticide-susceptible strains), in order to correct for variations in insecticide availability due to storage conditions for the nets.

2. STAGE 2 – EXPERIMENTAL HUT STUDIES

If the new LLIN product demonstrates significant increased efficacy compared to the standard pyrethroid-LLIN against all or most of the resistant strains tested in the laboratory, Stage 2 experimental hut studies should be initiated.

2.1. Objective

To demonstrate that the candidate LLINs (prepared according to WHOPES guidelines) are significantly better at inducing mortality and/or preventing blood-feeding than a standard LLIN (or reducing fecundity and fertility of the mosquitoes if a growth regulator is involved) against local highly resistant mosquitoes.

2.2. Site criteria

Experimental hut studies need to be conducted in areas where the mosquito population has high levels (RR > 10-fold) of well-characterized pyrethroid resistance. For data to be accepted, the resistance profile and species composition of the site must be determined immediately prior to, or at the same time as, the trial.
This profiling must include:

a. WHO diagnostic dose assays for pyrethroids (deltamethrin and permethrin as a minimum).\(^1\)

b. LC\(^{50}\)\(^2\) for all AIs incorporated into the net. A fully susceptible strain should be used as the standard for calculating the resistance ratio of the field population. (If An. gambiae s.s. is the local vector, the Kisumu strain should be used).

c. If a synergist is being tested, the effect of pre-exposure to the synergist on insecticide mortality needs to be recorded. For piperonyl butoxide (PBO) this should be a one-hour exposure to 4% PBO in a standard WHO tube bioassay.

d. A baseline of the species composition (including sibling species defined by molecular markers) of vectors entering the experimental huts prior to the study.

e. Cone bioassays testing onetime washed and regenerated pyrethroid-only LLINs with local mosquito vectors must be performed prior to the study.

f. At least 100 adult females (2–5-day-old non-blood fed, non-exposed to insecticides) should be preserved in an RNA stabilizing reagent (eg. RNAlater) at the start of the study for future follow up of resistance mechanisms, if required.

Note: Suitable study sites will have a vector population that has an RR > 10-fold for one or more pyrethroids at the LC\(^{50}\) level when compared to the standard Kisumu strain. Cone tests must also show > 50% survival of resistant mosquitoes against the standard pyrethroid-only LLIN. Tests undertaken in areas with lower level resistance cannot be used to substantiate product claims against highly pyrethroid-resistant populations.

2.3. Methods

The methodology follows WHOPES guidelines for testing LLINs at the experimental hut level\(^1\) and the same parameters are calculated (deterrency, induced exiting, blood-feeding inhibition, personal protection and mortality). If sterilizing properties are to be recorded, blood-fed mosquitoes from huts using both net types need to be kept alive and the fertility/fecundity recorded. Additional outcomes may be considered and introduced depending on the claim of the manufacturer.

Species composition of alive and dead mosquitoes should be determined if there are multiple sympatric vectors, in order to evaluate whether the net is equally effective against all.

Trials should be undertaken in at least three geographically distinct locations with different vector populations (eg. different transmission settings and/or resistance profiles) to assess whether the product is effective at multiple sites.

The trial must include comparison with a WHOPES-recommended LLIN.

http://apps.who.int/iris/bitstream/10665/80139/1/9789241505154_eng.pdf

\(^2\) LC\(^{50}\) should be determined using WHO procedures for determining intrinsic insecticidal activity, as outlined in the WHO Guidelines for Testing Mosquito Adulticides for Indoor Residual Spraying and Treatment of Mosquito Nets.
http://whqlibdoc.who.int/hq/2006/WHO_CDS_NTD_WHOPES_GCDPP_2006.3_eng.pdf
3. STAGE 3 – LARGE-SCALE FIELD TRIALS

The format of the community trials will depend on whether the mixture/combination LLIN functions through personal protection of the end user or relies predominantly on creating a community effect.

i. Fast-acting and repellent compounds will maintain personal protection of the end user and thus evaluation at a household level using a household randomized design will be sufficient.

ii. For all other modes of action, including slow action, epidemiological evidence will be needed due to a loss of personal protection for first in line products, or as determined by VCAG. A community-scale randomized controlled trial (RCT) design will be required for slow-acting or non-repellent1 insecticides or products which are expected to affect mosquito fecundity and/or fertility.

3.1. Study design for LLINs that work through personal protection

3.1.1. Objectives
To demonstrate that, under field conditions, the new product significantly reduces the number of blood-fed mosquitoes collected resting and exiting houses, compared to a pyrethroid-only LLIN.

3.1.2. Study methods
New products which offer personal protection can be tested at the household level with a household randomized control design. This type of trial is suitable, for example, for nets with a rapid acting insecticide plus a synergist.

3.1.2.1. Pre-trial considerations
Potential sites need to be characterized prior to trial to ascertain:

a. WHO diagnostic dose assays for pyrethroids2 (deltamethrin and permethrin as a minimum)

b. LC50 for all AIs incorporated into the net.3 The Kisumu susceptible strain should be used as the standard for calculating the resistance ratio of the field population if the local vectors are An. gambiae s.s.

c. If a synergist is being tested, the effect of pre-exposure to the synergist on insecticide mortality needs to be recorded. For PBO, this should be a one-hour exposure to 4% PBO in a standard WHO tube bioassay.

d. A 3-month baseline of the species composition (including form for An. gambiae s.s) of malaria vectors at the field trial site prior to the study, which should be a minimum of 3 months.

1 Pyrethroids lose their repellency action against pyrethroid-resistant populations and therefore combining a pyrethroid with a non-repellent insecticide or synergist would not allow a trial at household level to be a sufficient test.
e. A minimum of 100 mosquitoes should be tested in each case, for a–c above.
f. Cone bioassays on one-time washed and regenerated pyrethroid-only LLINs and local vectors must be performed prior to the study.
g. At least 100 adult females (2–5-day-old non-blood fed, non-exposed to insecticides) should be preserved in an RNA stabilizing reagent (e.g., RNAlater) at the start of the study for future follow up of resistance mechanisms, if required.

3.1.2.2. Trial procedures

After the baseline data above are collected, the candidate and standard net types should be randomly assigned to households and quarterly indoor and exit collections made over a transmission season. Mosquito densities will be compared between a reference pyrethroid LLIN (positive controls) and the candidate LLIN. Additionally, the mosquito densities should be noted before and after the intervention in indoor and exit collections, as well as the physiological status of female mosquitoes and any instances of delayed mortality.

Data will only be considered for trials that have been conducted in an area with documented >10-fold pyrethroid resistance, where the resistance status has been determined at the time of the trial.

3.2. Study design for LLINs that work only through community protection.

For LLINs that work at the community rather than the individual level and that do not offer personal protection, full-scale epidemiological trials will be needed until sufficient evidence has been generated to support the paradigm so a cluster randomized design will be applicable.

Indicators of epidemiological outcome could include: incidence of malaria through active case detection, passive case detection, serology, and/or point prevalence of infection. Entomological outcomes such as human landing catch, entomological inoculation rates and parous rates should also be considered.

The design and analysis of these trials should be based on methods appropriate for cluster randomized trials and standard errors and significance tests should be estimated accordingly.

In order to facilitate assessment and to standardize testing between products and between independent trials of the same product, SOPs are being developed and example trial formats will be made available with this document through VCAG.

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2 Pyrethroids lose their repellency action against pyrethroid-resistant populations and therefore combining a pyrethroid with a non-repellent insecticide or synergist would not allow a trial at household level to be a sufficient test.
APPENDIX 1

Standard insecticide-susceptible and insecticide-resistant strains used by industry for insecticide development and available as standards for testing via replace with Liverpool Insect Testing Establishment, Liverpool, UK. Well characterized strains from other sources may also be used (see section 1.3 above). Characterized strains from other institutions will be added to this list in due course and all the information will be made available and updated regularly on VCAG website.

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Country of origin</th>
<th>Phenotype</th>
<th>LC50 Deltamethrin (µg/ml)</th>
<th>Kdr</th>
<th>Ace</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kisumu</td>
<td>Anopheles gambiae</td>
<td>Kenya</td>
<td>Susceptible</td>
<td>0.020</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Kisumu Rdl</td>
<td>Anopheles gambiae</td>
<td>Kenya</td>
<td>Dieldrin resistant</td>
<td>To be determined</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Akron</td>
<td>Anopheles gambiae</td>
<td>Benin</td>
<td>Carbamate resistant</td>
<td>To be determined</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>VK7</td>
<td>Anopheles gambiae</td>
<td>Burkina Faso</td>
<td>DDT resistant</td>
<td>0.260</td>
<td>0.4</td>
<td>0</td>
</tr>
<tr>
<td>Tiassale</td>
<td>Anopheles gambiae</td>
<td>Côte d’Ivoire</td>
<td>Pyrethroid resistant</td>
<td>1.590</td>
<td>0.9</td>
<td>0.4</td>
</tr>
<tr>
<td>Moz</td>
<td>Anopheles arabiensis</td>
<td>Mozambique</td>
<td>Susceptible</td>
<td>To be determined</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>New Orleans</td>
<td>Aedes aegypti</td>
<td>USA</td>
<td>Susceptible</td>
<td>0.004</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>Cayman</td>
<td>Aedes aegypti</td>
<td>Grand Cayman</td>
<td>Pyrethroid, carbamate and DDT resistant</td>
<td>9.290</td>
<td>0.7</td>
<td>n/a</td>
</tr>
<tr>
<td>FuMoz</td>
<td>Anopheles funestus</td>
<td>Mozambique</td>
<td>Pyrethroid and carbamate resistant</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

kdr, knockdown resistance