



Revised Common Protocol for Uniform Evaluation of Public Health Pesticides including Bio-larvicides for use in Vector Control

2014



**Indian Council of
Medical Research**



**National Vector Borne
Disease Control
Programme**



**National Centre
for Disease Control**

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Research, New Delhi



National Vector Borne Disease
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Abbreviations

| | |
|------------------|---|
| ANOVA | Analysis of Variance |
| API | Annual Parasite Incidence |
| CDC | Center for Disease Control |
| CDS | Communicable Disease Surveillance |
| CHC | Community Health Centre |
| CIB | Central Insecticide Board |
| CPT | Complete Protection Time |
| CS | Capsule Suspension |
| DEET | N,N-Diethyl-meta-toluamide |
| ECOP | Environmental Codes of Practice |
| ED | Effective Dose |
| EDPT | Early Diagnosis and Prompt Treatment |
| EIR | Entomological Inoculation Rate |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| FT ₅₀ | First Takeoff |
| GCDPP | Global collaboration for development of pesticide for public health |
| HBI | Human Blood Index |
| HDPE | High-Density Polyethylene |
| HLC | Human Landing Catches |
| HTM | HIV/AIDS, TB, Malaria and Neglected Tropical Diseases |
| ICMR | Indian Council of Medical Research |
| IE | Inhibitor Emergence |
| IGRs | Insect Growth Regulators |
| IRS | Indoor Residual Spraying |
| ITN | Insecticide Treated Nets |
| IVM | Integrated Vector Management |
| KD | Knock Down |
| LD ₅₀ | Lethal Dose |
| LLINs | Long Lasting Insecticidal Nets |
| LSD | Least Significant Difference |
| MAP | Malaria Action Programme |
| MHD | Man-Hour Density |

| | |
|--------|--|
| MMF | Monomolecular Films |
| MPI | Monthly Parasite Incidence |
| MSDS | Material Safety Data Sheet |
| NCDC | National Centre for Disease Control |
| NIMR | National Institute for Malaria Research |
| NTD | Neglected Tropical Diseases |
| NTOs | Non-Target Organisms |
| NVBDCP | National Vector Borne Diseases Control Programme |
| PCR | Polymerase Chain Reaction |
| PHC | Primary Health Centre |
| PHPs | Public Health Products |
| PMD | Per Man-hour density |
| PPE | Personal Protective Equipment |
| PPM | Parts Per Million |
| PPS | Proportion Population Size |
| PVC | Polyvinyl Chloride |
| RDKs | Rapid Diagnostic Kits |
| SC | Suspension Concentrate |
| SOP | Standard Operating Procedure |
| SPR | Slide Positivity Rate |
| ULV | Ultra Low Volume |
| USM | Universiti Sains Malaysia |
| VCRC | Vector Control Research Centre |
| WG | Wettable Granules |
| WHO | World Health Organization |
| WHOPES | WHO Pesticide Evaluation Scheme |
| WP | Wettable Powder |

1. INTRODUCTION

Integrated vector management (IVM) is a universally accepted strategy for the prevention/control of vector borne diseases in a cost-effective and sustained manner. National vector borne diseases control programme (NVBDCP), the nodal agency of the Government of India for the control of vector borne diseases, has also endorsed the strategy. Among the available vector control options, chemical control remains to be the major method used in the control programmes especially in mitigating sporadic unpredictable outbreaks of vector borne diseases. Deployment of chemical control embraces the whole gamut of measures that include indoor residual spraying (IRS), application of larvicides and insect growth regulators (IGRs), distribution of insecticide treated nets (ITN)/ long lasting insecticide nets (LLINs) and an ever-lengthening list of household insecticide formulations for personal protection. In India, vector control measures recommended and practiced by the NVBDCP largely rely on site-specific chemical control, using insecticides belonging to different groups. A major impediment to this strategy has been the development of resistance by vector species to the insecticides, which necessitates replacement of the existing insecticides in the control programme with new insecticides showing adequate human and environmental safety. The NVBDCP has the ultimate responsibility to introduce new insecticides or insecticide formulations and insecticide treated/incorporated materials to the national control programme based on the results of scientific evaluation of the products. To facilitate the process, industries (national and international), National Centre for Disease Control (NCDC), institutes of the Indian Council of Medical Research (ICMR) and NVBDCP have to conduct laboratory and field trials to evaluate the insecticide compounds for their bio-efficacy and effectiveness on target and non-target organisms to arrive at the decisions. The products need to be evaluated in multi-centric mode at different sites with variable eco-epidemiology to ascertain their adaptation for control in diverse situations in the country. Further, it is mandatory that only the insecticides that are registered with the Central Insecticide Board (CIB) are to be used in the control programme. The WHO Pesticide Evaluation Scheme (WHOPES) is the only international programme aiming at promotion and evaluation of pesticides for public health use by providing technical assistance to the member countries and also encouraging the industries to develop more promising insecticides for the vector control programme.

1.1. Need for a common protocol for uniform evaluation

NVBDCP has been the nodal agency to select and introduce new public health products (PHPs) (which include insecticides/ insecticide formulations/ LLINs/ bio-larvicides) for vector control under national programme on the basis of their suitability and adaptability to Indian conditions assessed through multi-centric laboratory and field trials by the collaborating research organizations (ICMR/ NCDC). For the products to be approved by NVBDCP for use in national programme and to meet the mandatory requirement of CIB registration, it is necessary to generate data on entomological parameters and impact on disease incidence/prevalence. The WHOPES recommended pesticides are approved by the NVBDCP for use in national programmes only after large scale and multi-centric field testing/evaluation for efficacy and suitability to Indian conditions.

Although, general guidelines by WHOPEs for evaluation of insecticides are available, past experiences with insecticide evaluation have shown differences in the methodology adopted by different institutes and because of this a meaningful comparison of results generated at different sites becomes difficult for NVBDCP to decide the suitability of the products for Indian conditions. In order to avoid such difficulties, development of a common protocol for uniform evaluation of public health pesticides including bio-larvicides has become imperative. The trials conducted by different institutions at different sites following such common protocol will minimise the discrepancies in the methodology and thereby their results could be compared more meaningfully to facilitate the NVBDCP to arrive at a decision. Keeping this in view and also to be in line with the WHOPEs guidelines for insecticide evaluation, a common protocol was jointly prepared by the National Institute for Malaria Research (NIMR), New Delhi and the Vector Control Research Centre (VCRC), Puducherry in the year 2000, which has been revised/ updated by the Sub-Committee on revision of SOP and common protocol constituted by the DG, ICMR.

1.2. Procedure for evaluation of insecticides

The laboratory and field evaluations of insecticides are performed under three Phases.

Phase I (Laboratory evaluation)

Laboratory efficacy of new technical PHPs or their formulations is done under controlled conditions using laboratory-reared vector species. This phase includes studies on efficacy and persistence, diagnostic dosage and cross-resistance in vectors.

- Phase I evaluation may not be necessary for WHOPEs passed PHPs
- Sponsoring industries (national/international) has to provide data on human/ mammalian toxicity and environmental safety
- PHPs to be tested should have clearance from the respective institutional ethical committees.

Phase II (Small scale evaluation)

Small scale evaluation is carried out in the field or in simulated field condition to determine the application dosage and frequency of application. This phase provides sufficient information on efficacy in field conditions including safety of the insecticide to operators and inhabitants. It is also an opportunity to verify the effect of the insecticide on relevant non-target organisms, as given below. This phase suggests the suitability of the given PHP for testing in Phase III.

- Phase II evaluation of a product if already carried out in India under WHOPEs need not be repeated again in India.
- Informed consent should be obtained from the human volunteers associated with the evaluation

Non-target organisms (NTOs)

In order to maintain uniformity in generation of information, evaluation will be carried out against the following NTOs, depending on their prevalence in the test habitats/ areas.

Larvivorous fish: *Gambusia affinis*
Aplocheilus blockii
Tilapia mossambica
Poecillia reticulata

Water bugs: *Diplonychus indicus*
Notonecta sp.
Nepa sp.
Ranatra sp.
Anisops sp. (Back swimmer)

Dragon fly nymph
Chironomous sps larvae
Tadpoles
Silk worm moth (*Bombyx mori*)
House crickets (*Acheta*, *Gryllus* sp)
Honey bees (*Apis mellifera*/ *Apis indica*)

Phase III (Large scale evaluation)

Evaluation in this phase is done at the Phase II recommended dosage on a large scale [village(s) scale] against disease vectors prevalent in the area. This phase includes assessing the impact on entomological parameters, disease incidence/ prevalence and relevant non-target organisms and also evaluating community acceptability and operational safety.

- Phase III evaluation should be carried out at least in three eco-epidemiological settings (multi-centric), preferably in two high endemic areas and one low endemic area.
- Informed consent should be obtained from the human volunteers associated with the evaluation

Quality control:

Quality control data need to be generated for all the products to be evaluated by any accredited laboratory in the country.

NVBDCP Guidelines on ‘Proper storage, safe handling and disposal of insecticides’ and on ‘ITNs and LLINs’ (available at www.nvbdcplnic.in) should be followed for delivery, distribution and disposal of insecticides, packages, insecticide treated materials including long lasting insecticidal nets.

2. PROTOCOLS FOR EVALUATION OF INSECTICIDES

2.1. Indoor Residual Spraying (IRS)

Indoor residual spraying (IRS) is one of the effective vector control tools. In India, it is extensively used for the control of malaria and kala-azar. Indoor resting (endophilic) mosquitoes effortlessly pick up the lethal dosage of insecticide through their tarsal contact and such lethal contact reduces the longevity of the infected vectors resulting in the interruption of disease transmission. IRS is the application of chemical insecticide formulations such as wettable powder (WP)/ capsule suspension (CS)/ suspension concentrate (SC)/ wettable granules (WG) in the habitat of vector resting. The effectiveness of IRS depends on the following criteria:

- Endophily and endophagy and also partial endophily of mosquitoes (mosquitoes which rest indoors for some time before and/ or after blood meal)
- Adequate coverage of sprayable surfaces in the habitats such as walls, eaves, ceiling/ roof and other potential resting places of disease vectors
- Residual activity of the insecticide formulation throughout the transmission period

The insecticides which are evaluated for the first time (new insecticides), the standard specifications of the compound should be provided by the sponsoring agency. The sponsoring agency should also provide toxicological indices on safety for humans and non-target organisms especially against domestic pet animals. (In other words, the material safety data sheet (MSDS) for the new insecticide should be provided). Evaluation should be done in three Phases, I, II and III.

The new insecticides that showed promising activity in the laboratory trial (Phase I) will be considered for Phase II and subsequently Phase III evaluation. The insecticides passed by WHOPES will be taken directly for Phase II and Phase III evaluation.

2.1.1. Laboratory evaluation (Phase I)

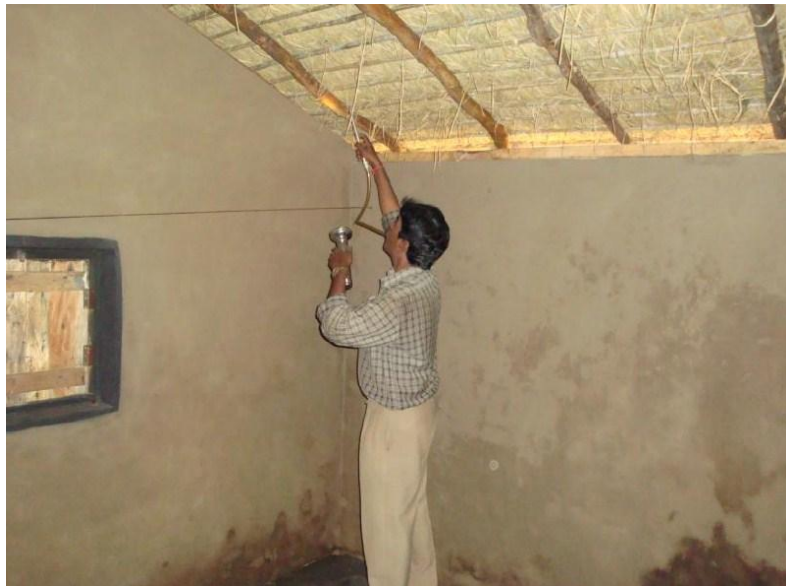
Duration: 3 months

Objectives

- To find out the intrinsic toxicity of the given insecticide against the target vector species by determining LD₅₀ and LD₉₀
- To determine the diagnostic dosage for monitoring resistance to the insecticide and cross-resistance to other insecticides in the field
- To assess irritant and excito-repellent properties of the insecticide by determining ('Time to first take off') FT₅₀ and FT₉₅ after exposure to treated substrates.
- To assess the efficacy and residual activity of the insecticide



Indoor residual spraying



Collection of mosquitoes resting indoors

2.1.1.1. Intrinsic toxicity

Objective

- To determine the intrinsic toxicity of an insecticide to a target species

This is done by the topical application of an active ingredient to isolate toxicity from confounding effects resulting from insect behavior (WHO/CDS/NTD/WHOPES/ GCDPP/ 2006.3).

Method of testing intrinsic toxicity

- The technical grade insecticide is dissolved in acetone, a highly volatile organic solvent that remains on the insect cuticle only for a short time, to prepare topical solutions. (the dosage is expressed in nanograms of active ingredient per mg of body weight of live mosquito).
- Fifty non-blood-fed susceptible female mosquitoes are weighed initially to determine the average live-weight.
- A constant volume of 0.1 μ l is added on the pronotum using a pipette. Adding larger volumes should be avoided as it may cause higher mortality due to solvent toxicity.
- After testing with wide range of concentrations, a narrow range of at least five concentrations causing a mortality range from 5% to 99% (preferably 2–3 dosages <50% and 2–3 >50%) should be selected and used per test. A total of 50 susceptible, non-blood-fed, 2–5 day-old female mosquitoes are tested at each concentration.
- The mosquitoes are anaesthetized (using CO₂ for 30 seconds) and placed on a plate under cooling at 4 °C and thereby the anesthesia condition is maintained during the manipulations.
- For the treatment group, two batches of 25 mosquitoes are tested at each concentration of the insecticide.

Using a suitable applicator, 0.1 μ l of the insecticide solution of the required concentration is deposited on the pronotum of the females., running a parallel control of two batches of 25 female mosquitoes treated at 0.1 μ l of pure acetone.

After dosing, the females are transferred into clean holding cups provided with 10% sugar solution soaked on cotton wool and held for 24 hours at 27 ± 2 °C temperature and 80 ± 10 % RH to record the mortality as a result of topical application.

- The test is repeated three times testing separate batches of reared mosquitoes and the results of the three tests are combined for statistical analysis.
- Whenever the test is repeated fresh insecticide dilutions should be prepared and used.
- Log-dose probit regression (Finney, 1971) is used to analyze the relationship between dose and mortality. LD₅₀ and LD₉₀ and their 95% confidence limits are determined using commercial software. If mortality exceeds 20% in controls, the test is rejected. If mortality in the controls is between 5% and 20%, the treated mortality is corrected to the control mortality using the Abbott's formula as given below:

$$\text{Mortality (\%)} = \frac{X - Y}{100 - Y} \times 100$$

Where

X = percentage mortality in the treated sample(s) and

Y = percentage mortality in the control.

- The probit mortality per log dose regressions for two insecticides could be compared using a parallelism test (WHO/CDS/NTD/ WHOPES/ GCDPP/ 2006.3).

2.1.1.2. Diagnostic dosage

The diagnostic dosages to be used for susceptibility test are those recommended by WHO to detect or monitor the presence of resistance in the target vector species to a given insecticide.

Preparation of insecticide impregnated papers

- Diagnostic dosage is determined by exposing the target mosquito species to a graded series of dosages of insecticide (technical grade) impregnated on filter-paper.
- Two ml of acetone, and mixed with a non-volatile carrier such as silicon oil (e.g. BDH Dow Corning® 556) or Risella® (Shell) or olive oil (according to the insecticide to be tested) is applied to rectangular pieces of Whatman® No. 1 filter-paper measuring 12 x 15 cm. .
- The carrier oil allows to form a stable, thin and homogeneous layer of the insecticide on the filter-paper and also prevents crystallization of active ingredients.
- Since acetone is volatile, the concentration of the insecticide is normally expressed as % of active ingredient (ai) per unit volume of carrier oil on the filter-paper.
- Filter-papers are impregnated with 3.6 mg/cm² of the carrier oil, i.e. 648 mg/paper or 0.66 ml/paper for silicon oil (having a density of 0.98). A filter-paper impregnated at 1%, contains 6.6 mg of technical insecticide, or 367 mg/m².
- The filter paper is impregnated by pipetting the insecticide solution evenly on to the paper pinned on a cardboard.

- The papers after impregnation are air dried for 24 hours and used for testing. The impregnated paper should not be used more than five times (WHO, 1998).
- The adult susceptibility test (WHO tube test) method is described in Box 1.

Determination of diagnostic dosage

Mosquitoes are exposed to graded series of concentrations of the insecticide impregnated on papers. (The detailed procedure of testing following the WHO tube method is given in Box 1). Concentrations should be chosen in such a way that at least one concentration gives 100% mortality, at least two give between 50% and 99% mortality, and at least two give between 5% and 50% mortality.

The concentration/ mortality relationship is determined on three replicate batches. The results are then combined to produce a log dose/ probit mortality regression line from which the LD₉₉ is estimated. The diagnostic dosage corresponds to twice the lowest concentration that kills 100% of the exposed mosquitoes.

2.1.1.3. Residual effect on substrates

This is to identify the target dosages for Phase II trial in experiment/ village huts based on residual activity of the sprayed insecticide deposits on different surfaces. Residual activity is tested on different pre-fabricated substrates such as mud, brick, thatched, cemented, tin, etc. The substrates are 40 cm X 40 cm prepared on wooden frames. A minimum of seven replicates per dosage for each substrate are prepared, at least four for bioassay and three for initial chemical analysis, selected at random. For chemical analysis, three samples from each of the three substrates are used. Bioassays are done in the beginning to determine the lowest concentration causing 100% mortality. All the seven replicates for each substrate are sprayed with the insecticide to make a homogenous residual deposit of the desired range of concentrations (2-4 times of the lowest concentration that causes 100% mortality) per unit area using a Potter Spray Tower[®], the internationally recognized method for laboratory spraying (WHO, 2006). All the treated substrates are stored unsealed under controlled conditions of temperature

Box 1: Adult susceptibility test (WHO tube method)

Susceptibility test is conducted using the WHO test kit and method (WHO 1998). The test kit and papers impregnated with insecticides at the WHO recommended diagnostic dosage could be obtained on payment from the Vector Control Research Unit, School of Biological Sciences, 11800 Universiti Sains Malaysia (USM), Penang, Malaysia (who makes on behalf of WHO).

Kit: The test kit includes green dotted (holding tube) and red dotted (exposure tube) plastic tubes (of 125 mm in length and 44 mm in diameter), with each tube fitted at one end with a 16 mm mesh screen, slide-units with screw cap on either side with a large orifice for transferring mosquitoes and a small orifice for introducing mosquitoes by aspirator; copper and steel clips; instruction sheet; log-probit papers; report forms; glass aspirators with 60 cm rubber tubing and mouth piece; roll of adhesive tape and white paper sheets (12 x 15 cm).

Method: Tubes with green dot should be used for holding of mosquitoes and for control exposures. Tubes with red dot should be used for exposures to insecticide papers. The green dot tube should be lined from inside with a plain paper fastened with a steel clip and later fixed to the slide by threading into screw cap. As needed, the required number of green dot tubes are lined from inside with insecticide-control papers duly fastened with steel clips and red dot tubes lined with insecticide impregnated papers of the designated dosage and fastened with copper clip.

Tests should be performed preferably with 2-5-day old sugar fed females of laboratory strain or 2-5 day old sugar fed F1 female progeny of field-collected adults or females emerged from the immature collected from field. Where only field collected adults can be used, their physiological status (i.e. unfed, blood fed, semi-gravid, gravid) should carefully be recorded (WHO, 1998).

Batches of 20-25 non-blood-fed female mosquitoes, aged 2–5 days, are introduced into each holding tube (with a green dot) through the small orifice on the slide and closed and held for one hour at $25\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ and $80\% \pm 10\%$ RH to acclimatize. The holding tubes are appropriately labeled with locality, species tested, etc. and provided with glucose source for feeding. After the holding period to observe for injured and/ or dead mosquitoes, if any, green dot tubes with insecticide-control papers and red dot tubes with insecticide impregnated papers are screwed to the respective holding tubes. The mosquitoes are transferred by gentle blowing to the tubes with insecticide-control papers and with insecticide impregnated papers and the tubes are held vertically for one hour under subdued light. During the exposure time, glucose source should be removed. At the end of the exposure time, the mosquitoes are gently blown back in to the respective holding tubes which are placed vertically in a dark place for 24hours with sucrose solution at $25\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ and $80\% \pm 10\%$ RH. Dead mosquitoes are counted after 24 hours. A total of 100 mosquitoes (four - five) replicates containing 25 or 20 mosquitoes in each tube) are used for each test concentration and for the control. Results are expressed as percentage mortality after 24 hours and corrected for any control mortality.

After each exposure, the test kit should be washed with soap and clean water and dried.

($30\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$), relative humidity (80%), air circulation and ambient light cycles until they are used for testing. Residual activity is determined by cone bioassays on four replicates per dosage for each substrate exposing the target vector species for 30 minutes and recording the mortality after 24 hours holding period, following the method given in section 2.1.3.6. After the insecticide application, bioassays on the treated substrates should be done initially for one

week and subsequently at fortnightly/ monthly intervals until the mosquito mortality drops below 80%. From this assessment, three to five best dosages will be selected for Phase II evaluation.

2.1.1.4. Irritant or excito-repellent properties

One of the important properties of an insecticide is its irritancy. This property needs to be considered during evaluation as it alters the time of the mosquito tarsal contact with the treated surface. Irritancy of an insecticide is studied by releasing mosquitoes in to a WHO specified cone (made of PVC) fixed on an insecticide treated surface. The mouth of the cone is closed with a polyethylene plug. The released mosquitoes in the cone remain in contact with the insecticide treated surface since they do not generally prefer to rest on PVC cone or polyethylene plug.

The insecticide irritancy is first assessed using a filter paper impregnated with the technical grade of the given insecticide at the diagnostic dosage as described in section 2.1.1.2. If there is any significant irritancy with the treated filter paper compared to the control (paper impregnated with acetone and silicon oil only), further tests are carried out with appropriate formulations of the insecticide on substrates commonly used for making houses/ shelters (mud, cement, plywood, thatch).

The selected surfaces are sprayed with the recommended dosage (i.e. the lowest one causing $\geq 80\%$ mortality for longer duration) of the insecticide. For each test, susceptible, non-blood fed, 2-5 days old female mosquitoes (50 numbers) are individually introduced in to plastic cones. After allowing 60 seconds for the mosquitoes to settle down, the time elapsed between 'first landing' and the 'next take off' of the mosquito is recorded as FT. Mosquitoes are then grouped by classes of first take off time (0-1 s, >1-2 s, >2-4 s, >4-8 s,>128-256 s) and FT₅₀ and FT₉₅ (the time before 50% and 95% of the mosquitoes take off) are calculated based on cumulative frequencies using probit analysis. Mosquitoes that do not take off at least once during the 256 seconds exposure (test period) are discarded. An insecticide that is well known for its irritancy (e.g. Permethrin) should be used as a positive control wherever possible.

2.1.1.5. Cross resistance

To assess the cross resistance in mosquito vectors that have developed resistance to other insecticides in use under vector control programme, susceptibility tests will be carried out using WHO test kit as described in section 2.1.1.2.

The susceptibility status of vector species should be categorized as per the WHO criteria: susceptible- 98 to 100% mortality, verification required- 81 to 97% mortality, resistant- < 80% mortality. Data should be recorded in the format given in Table 1

Table 1. Insecticide susceptibility test (WHO tube method)

Village Sub-centrePHC District

Insecticide (%)..... Impregnation dateNo. of times paper used

Date of Test.....Temp: Min MaxHumidity: MinMax

Test speciesLab/F1/Field collected species

Exposure time Minutes

| Replicate | No. exposed** | Number knocked down in 1 h | No. dead after 24 h | % mortality | Corrected % mortality# | number* |
|--|---------------|----------------------------|---------------------|-------------|------------------------|---------|
| Test 1 | | | | | | |
| Test 2 | | | | | | |
| Test 3 | | | | | | |
| Test 4 | | | | | | |
| Control 1 | | | | | | |
| Control 2 | | | | | | |
| <p>*Separate row for each replicate; **20-25 mosquitoes/replicate; #After Abbott’s formula when mortality in control replicates is between 5 and 20% (<5% no correction is needed and > 20% test to be discarded & repeated).</p> $\text{Corrected mortality (\%)} = \frac{(\% \text{ Test mortality} - \% \text{ Control mortality})}{(100 - \% \text{ Control mortality})} \times 100$ | | | | | | |

2.1.2. Small-scale field trials (Phase II)

Objectives

General

To assess the efficacy and residual activity of insecticides against wild population of the target vector species

Specific

- To determine the optimum application dosage of the insecticide to be used for Phase III evaluation.
- To measure the efficacy of insecticides in terms of mortality (immediate and delayed) and residual effect
- To study the impact on the behaviour of mosquitoes (deterrence, blood feeding inhibition and induced exophily)
- To record the ease of application and perceived side-effects by the spray-men and the inhabitants during application and use.

2.1.2.1. Trial in experiment huts

Duration: 6 months (preferably during the season when the vector density is higher)

Number of dosages to be used: 3-5

Efficacy of insecticides can be determined only where entry and exit of mosquitoes are monitored and scavenging of knocked down or dead mosquitoes is prevented. Such conditions can be obtained only in experimental huts.

2.1.2.1.1. Experimental hut study design

The experimental hut consist of a single room with four windows; size of each window should be 0.45 x 0.45 m, grilled with wooden planks fixed horizontally in tilted position one above the other leaving a gap of 1 cm between two planks through which mosquitoes could enter into the hut but could not exit. There are two windows on the front door side and one on each of the sides and a screened (using nylon mesh) verandah (verandah trap) at the backside. The dimensions of the huts resemble almost to those of the village huts (Length 3 m, width 3 m and height 2.5 m) have brick walls with cement plastering and thatched roof, above which there is tin sheeted roofing for protecting the thatched roof. There should not be space between the thatched ceiling and tin-roof. The huts are constructed one foot above the ground level on a platform made up of brick and cement. The platform has a water-filled moat (6' depth x 6' breadth) all around to deter entry of scavenging ants. The moat is made at two feet away from the hut walls, except on the back side of the hut where it is at 1.5 ft away from the base of the verandah trap. At the centre of the hut, the roof is at a height of 2.5 m and near the wall the height is 2 m; this difference in height is to maintain a slope of the roof. The eave on the backside (facing towards east) has a gap of 1-2 cm and through this gap mosquitoes could exit, but those mosquitoes will be collected in the verandah trap. There is one wooden door of 0.75m x 1.5m facing towards west. For small scale field trials, preferable, several huts are required to compare different treatments simultaneously. A minimum of four replicates (four huts) per treatment arm and an equal number of control huts are to be used.

2.1.2.1.2. Assessment prior to hut trial

Prior to the hut trial, assessment is essential to ensure that the huts are comparable in their attractiveness to the target mosquito species and also to ensure that the huts are not contaminated with insecticide.

For acclimatization and to attract mosquitoes into the experimental hut, an adult volunteer enrolled for this purpose should sleep (preferably under an untreated mosquito net) in each of the huts from dusk to dawn for a period of 15 days.

Subsequently, the suitability of the experimental huts for conducting the trial is assessed based on the following criteria over a period of one month prior to starting the trial.

- 1) ***Indoor resting of mosquitoes:*** The resting mosquitoes are collected from the experimental huts in the morning hours weekly twice, keeping equal intervals between the two successive collections. In parallel, mosquitoes should also be collected from the randomly selected village huts (number should be equal to the number of experimental huts). The mosquitoes are identified to species and counted. Per man-hour density (PMD) (number of female mosquitoes collected/man-hours spent) of the target vector species is calculated for the experimental and village huts and compared between the two. Statistically equal density in experimental and village huts or a higher density in experimental huts than the village huts indicate the suitability of the experimental hut.
- 2) ***Tightness of huts (from recovery rate):*** Recovery rate (number of mosquitoes recaptured/total number released X 100) is used to verify the tightness of the experiment huts. Around 75 (depending on availability) fully-fed field collected female mosquitoes are released during one evening into each experimental hut and after the release the huts need to be closed. The next day morning mosquitoes are recaptured. A recovery rate of at least 70% ensures the tightness of the hut. The recovery rate should be assessed on a minimum of five occasions.
- 3) ***Absence of scavengers:*** To ensure the absence of scavengers inside the experimental huts, four batches of 25 dead mosquitoes are kept on the floor including verandah (in four corners) of each hut in the evening and the number present in the next day morning is recorded. Such observations should be made on eight occasions, twice a week during the four weeks.

Each experimental hut trial should have a negative control and, if possible, a positive control. For evaluating an insecticide for IRS, the negative control involves only a sleeper without insecticide treatment or with the formulation minus the active ingredient. This is relevant in situations, where the inert ingredient of the formulation or substrate may exert an effect by itself. For a positive control, an insecticide commonly used in the country at a recommended dosage will be used.

The hut trial should be a blinded one. All field staff including supervisors engaged in the trial be blinded to the allocation of treatments to avoid bias during the evaluation. Usually double-blinding of senior investigators and the field staff (who are involved in implementation) is desirable, if not, the minimal requirement is single blinding of the implementing personnel and supervisors.

2.1.2.1.3. Rotation of sleepers

For IRS trials, treatments cannot be rotated, and hence it is necessary to demonstrate that there is little or no variation in the attractiveness of huts during pre-trial assessment (this also illustrates the importance of optimum positioning of huts during construction). The sleepers should be rotated between huts so that every sleeper is allocated to each hut-treatment an equal number of times. Practically, sleepers will have to be rotated daily between the huts.

2.1.2.1.4. Ethical clearance

The trial proposal should be submitted to the respective ethical institutions and authorities and clearance should be obtained before undertaking the trial. Informed consent should be obtained from the volunteers involved in the study (Annexure 1). Proper medicare, including chemoprophylaxis (as per the national guidelines), should be provided to the volunteers engaged for the trials.

2.1.2.1.5. Safety and precautions in implementation

During the trials, adequate safety precautions and the necessary protective measures should be strictly adhered. Guidelines for the treatment of intoxication and antidote should be available in the trial site and a responsible person should have the access to them.

Prior to initiation of any trial, it should be ensured that the experimental huts are completely renovated and cleaned. If the hut is already sprayed with an insecticide, the sprayed surfaces should be replaced and absence of contamination needs to be demonstrated by conducting suitable bioassay tests.

The insecticide formulation should safely and correctly be applied in the huts following the WHO guidelines (WHO, 2003). Conventionally, the walls, ceiling, eaves and doors are sprayed, but this may be altered according to the nature of the treatment and the manufacturer's recommendations. As IRS causes a higher degree of contamination to the huts, it will be necessary to remove and replace the door, substrates and ceiling material between trials.

2.1.2.1.6. Assessment of spray quality

To assess the quality of spraying, at least four Whattman filter papers (each paper 15cm X 15cm) leveled properly will be struck on the walls (one on each wall of the hut) of each experimental hut, before spraying, and removed after complete drying. The papers will be wrapped in aluminum foils and subjected to analysis for insecticide content. The chemical analysis results are combined for each substrate to provide the average concentration of insecticide (in mg/m²).

2.1.2.1.7. Evaluation

2.1.2.1.7.1. Dosage determination and residual activity assessment

The residual activity of the target dosages (as determined in Phase I trial) is assessed by conducting bioassays at regular intervals, preferably on day 1, day 7 post-spraying and thereafter weekly/ fortnightly, using WHO cones. Batches of 10 non-blood-fed, 2–5 days old mosquitoes are released in to each cone and exposed for 30 minutes on each of the walls of each hut and on the ceiling. Wherever, it is not practical to use non-blood fed 2-5 days old (F1 females) mosquitoes for the assay, wild caught blood-fed female mosquitoes may be used.

The duration (number of weeks/ months) up to which the mortality was above 80% (after 24 hours holding period), the cut of level is recorded.

While selecting a dosage for testing, the necessary safety aspects should be taken in to account.

2.1.2.1.7.2. Air-borne toxicity of insecticide

The fumigant property of insecticides is assessed by estimating the mortality of mosquitoes kept in wire gauze cages that are allowed to hang from the ceiling for 4–8 hours up to a maximum of 12 hours, at different distances from the sprayed surfaces. Five to 10 cages are placed in each hut and 25 non blood-fed female mosquitoes are released in to each cage.. Parallel controls should be maintained by placing mosquito cages in unsprayed huts. After transferring to clean cages, the mosquitoes are kept for 24 hours' observation and mortality, if any, is recorded. The air-borne toxicity is assessed by comparing the treated mortality in comparison to the controls.

2.1.2.1.7.3. Efficacy and impact on vector behaviour

When the trial is conducted in experiment huts, the volunteers involved for sleeping in the huts should carefully follow the instructions given by the research team supervisor. The sleepers should follow a standard time schedule to enter the huts in the evening for sleeping and remain inside until a standard time in the morning. Periodically, the research team supervisor, with the help of a local volunteer (or a person nominated by the village head) should make a check at night (without intruding in to their privacy) to ensure that the instructions are being followed by the volunteers sleeping in the huts. While the volunteers are inside the huts for sleeping, it should be ensured that the windows are kept open.

Mosquito collections

In the evening, before the volunteers occupying the hut, its room and verandah should be cleaned and white cloths spread on the floor of the hut including verandah. The verandah trap be furnished with cotton pads soaked in 5% glucose solution to reduce the risk that unfed female mosquitoes exiting in the night would die of starvation.

The next morning, the windows are closed and the dead mosquitoes found on the floor sheet are picked up using forceps and placed in cups provided with moist cotton wool, and then the white cloths are removed from the floor. The resting alive mosquitoes are collected separately from the veranda, room and net (if present) using aspirators and flashlights. All mosquito specimens collected from each part of the hut are kept separately, labeled, brought to the laboratory, identified to species and classified according to their gonotrophic condition (unfed, fed, half gravid, gravid). The live-caught females, provided with sugar solution, are kept on observation for 24 h to record delayed mortality, if any. Since, it would be difficult to maintain controlled conditions during holding of mosquitoes as strictly as done in Phase I trial, humidity and temperature should be controlled within tolerable limits either by using insulated containers for holding or wrapping wet towels around the holding cages.

Mosquitoes are collected in the huts, twice a week after spraying for a period until the density of the vector mosquitoes decline to a minimum level (based on the density of vectors in the control huts) due to seasonal effect. Data must be carefully recorded on the prescribed sheets.

The data collected from the replicates of each treatment should be compiled and consolidated to assess the four indicators of spraying efficacy and mosquito behavior in response to the treatment as described below.

2.1.2.1.7.4. Safety and operational issues

Spray men and other persons who handled the insecticides should be enquired about adverse effects, if any, perceived by them. This information would be useful to decide whether the given insecticide is suitable for testing at Phase III. Information should be collected on ease of application (mixing, dilution of insecticide and spraying) from the spray operators.. The volunteers who sleep in the huts should also be enquired regularly during the trial period about the perceived side-effects, if any, by them. In case of any such complaint, the researcher should immediately attend to it and ensure that they are relieved of discomfort.

2.1.2.1.7.5. Data analysis

Indicators

The efficacy of an insecticide used for indoor residual spraying is generally assessed using four indicators such as deterrence, induced exophily, blood-feeding inhibition and mortality. The data collected from the treated huts are compared with that from the untreated control huts for calculating these indicators.

1. The total number of female mosquitoes collected in the hut and verandah is the entry rate. Certain types of repellent insecticide possess deterrent effect causing a reduction of entry rate (deterrence) , probably because mosquitoes could detect the insecticide vapour or dust before they enter a treated hut.
2. The induced exophily or excito-repellency is estimated from the exit rate, which is the proportion of female mosquitoes collected in the verandah trap in comparison to the total number collected in the hut and verandah..
3. The percentage of blood-fed female mosquitoes among the total number collected in the hut (room + verandah) is the blood-feeding rate. The reduction in the blood-feeding rate in the treated hut compared to that in the control hut will give the blood-feeding inhibition caused by the insecticide.
4. The mortality rate (total mortality) is the proportion of female mosquitoes found dead in the hut immediately after spraying (immediate mortality) and 24 hours later (delayed mortality). The insecticide-induced mortality rate is calculated from the difference in mortality between a control hut (natural mortality) and a treated hut.

If an insecticide sprayed in the hut has considerable deterrent effect preventing significant number of mosquitoes from entering the hut, the values obtained from proportions blood feeding or killed by the insecticide spraying may not estimate the full personal protective effect. Therefore, in an experimental hut study, the personal protective effect of a treatment is determined from the reduction of the number of blood-fed mosquitoes in the sprayed hut in

comparison to the number blood fed in the control hut. The following formula is used to calculate the personal protective effect (WHO, 2006):

Protective effect (feeding inhibition) (in %) = $100 \times (B_c - B_t) / B_c$,

Where, B_c is the total number blood-fed in the control hut and
 B_t is the total number blood-fed in the sprayed hut

Similarly, the overall insecticidal effect of spraying should take into account that significant numbers were deterred and not killed by the insecticide spraying. It is estimated using the following formula and expressed as a percentage (WHO, 2006):

Overall insecticide effect = $100 \times (D_t - D_c) / E_c$,

Where, D_t : total number of mosquitoes dying in the sprayed hut,
 D_c : total number dying in the control hut and
 E_c : total number entering the control hut.

Statistical analysis

Prior to treatment, it should be ensured using an appropriate statistical test that there is no significant difference between huts in terms of attractiveness to mosquitoes.

Since, day to day entry of mosquitoes (distribution from day to day) is likely to be over dispersed and fits a Poisson distribution with variance equal to mean, Poisson regression analysis or a non-parametric test such as Kruskal-Wallis or Wilcoxon rank-sum test should be used (WHO, 2006).

The number of mosquitoes entered the huts (entry), the proportion of mosquitoes exit early (exit), the proportion died within the hut (mortality) and the proportion of blood-fed may be compared by species and analyzed using Poisson regression for numeric data and logistic regression for proportional data (e.g. Stata 6 Software). The clustering of observations made in one hut-night, and controlling for any variation between huts and sleepers, needs to be controlled for. Comparisons between treatments are made by successively dropping treatments from the overall comparison. This process allows each treatment to be compared with every other one. As a less powerful but valid alternative, the numbers of blood-fed and dead mosquitoes and overall totals collected from each hut may be compared using the nonparametric Kruskal-Wallis test (WHO, 2006).

2.1.2.2. Trial in village huts

Duration: 6 months

Dosages to be used: 3-5

Wherever, construction of experimental huts is not feasible, the phase II evaluation may be carried out in the existing village huts with minor modifications with the consent of the respective household heads.

2.1.2.2.1. Selection of dosages for application

From the residual activity assay results (Phase I laboratory trial) as described under section 2.1.1.3., three to five best dosages, flanking both sides of the LD99 values in Phase I. For example, if the calculated dosage is 2.0 (given units), the dosages for testing would be 1.0, 2.0, 3.0, 4.0 and 5.0 (given units) are selected for conducting phase II evaluation in the huts.

2.1.2.2.2. Selection of study area

Study area should be selected in consultation with respective State/ District health department and based on logistics. In case the study is proposed to be carried out in two or more villages, comparable villages in terms of vector density and eco-type should be selected. Vector density should be ascertained prior to the trial to select comparable villages. The houses with considerable vector density should be selected for the trial.

2.1.2.2.3. Selection of houses

A minimum of four replicates (four houses) per treatment arm and an equal number of control houses are to be included for the trial. Accordingly, in the selected village(s), a total of 24 houses (four houses for each of the five dosages and four control houses) should be selected. These houses should be designated for interventions among six arms (five treated arms and one control arm). Each house should be marked with the arm code and insecticide dosage code. The houses in each arm should be distributed equitably among the dosages of spraying and control (negative control) as shown in Table 2. In case of a request for comparison with an insecticide currently in use (positive control), additional four houses should be selected (total 28 houses) and distributed as shown in Table 3. Informed consent should be obtained from the head of the households for inclusion of their house for the trial and also to implement minor alterations in the structure of their houses, if needed.

No other vector control intervention should be undertaken during this phase of trial in the selected villages. Alternatively, villages without insecticide spraying by the programme may be selected as controls. In case the ongoing spraying is suspended during the trial, disease surveillance mechanism should be strengthened for providing protection from the disease to the villagers (as only a few houses are selected for spraying as per the evaluation criteria). Where feasible, hamlets or small villages having the required number of houses for the evaluation can be selected.

Table 2. Distribution of houses in arms with different dosages

| Replicates | Arms | | | | | |
|------------|--|----------|----------|----------|----------|-------------------------------------|
| | Houses with (candidate) insecticide spraying | | | | | Houses without insecticide spraying |
| | Dosage 1 | Dosage 2 | Dosage 3 | Dosage 4 | Dosage 5 | Control |
| 1 | House 1 | House 2 | House 3 | House 4 | House 5 | House 6 |
| 2 | House 7 | House 8 | House 9 | House 10 | House 11 | House 12 |
| 3 | House 13 | House 14 | House 15 | House 16 | House 17 | House 18 |
| 4 | House 19 | House 20 | House 21 | House 22 | House 23 | House 24 |

Table 3. Distribution of houses in the arms with different dosages with additional houses for spraying with comparison insecticide (positive control)

| Replicates | Arms | | | | | | |
|------------|--|----------|----------|----------|----------|-------------------------------------|--|
| | Houses with (candidate) insecticide spraying | | | | | Houses without insecticide spraying | Houses with spraying of comparison insecticide |
| | Dosage 1 | Dosage 2 | Dosage 3 | Dosage 4 | Dosage 5 | Control 1 | Control 2 |
| 1 | House 1 | House 2 | House 3 | House 4 | House 5 | House 6 | House 7 |
| 2 | House 8 | House 9 | House 10 | House 11 | House 12 | House 13 | House 14 |
| 3 | House 15 | House 16 | House 17 | House 18 | House 19 | House 20 | House 21 |
| 4 | House 22 | House 23 | House 24 | House 25 | House 26 | House 27 | House 28 |

Spraying of insecticide should be conducted by the investigators in collaboration with the PHC Medical Officer and District Malaria Officer with intimation to other health personnel of the programme in the state. For Phase II trial, spraying of the candidate insecticide should be done in the selected villages during the period when routine spray operation is carried out in the other villages by the NVBDCP either during first or second round (Box 2).

2.1.2.2.4. Spraying of houses

Spraying of houses is done with the standard equipment (knapsack lever operated or compression sprayer or stirrup pumps) following the spray norms (Box 3 & 4, Table 4) in use in the routine vector control programme. The technique of spraying should be the same as that is in regular use in the area. Care should be taken to spray the houses with complete coverage and proper dosage dispersion. Inhabitants should be informed in advance about the preparations to be done for spraying and precautions to be taken after spraying. This can be better accomplished with the help of local panchayat/ opinion leaders, school teachers, religious leaders and others. Spray men should be given orientation on spraying technique before spraying and on necessary precautions to be followed to protect themselves and the inhabitants from contamination. The required safety measures need to be ensured by the supervisory staff during the operation as given in Box 5. Good quality spray should also be ensured by the supervisor (Box 6). Spraying should be carried out under strict supervision and mopping up of spraying, if needed, should be done immediately in a day or two. Any lapse in the spraying will seriously affect the results of the trial. Coverage and other related details of spraying should be recorded in the proforma as given in Table 5.

2.1.2.2.5. Assessment of the quality of treatment

Refer to section 2.1.2.1.6. for assessment of the quality of spraying by chemical analysis.

2.1.2.2.6. Assessment of residual activity on different surfaces

Residual activity is determined using cone bioassays (WHO 1981) on different wall surfaces available in the study area, viz. cement, mud, thatch, tin, etc. Houses sprayed with different doses of the insecticide, with different surfaces should be selected for the cone bioassays. Similarly, surfaces should be identified in control houses. On the selected surfaces, areas of 1 sq ft should be marked with pencil. At least 4 squares should be marked for a given dosage of insecticide for each type of surface. Not more than 2 squares should be selected in one house for a given type of surface. At least 2 squares for each type of surface should be marked on unsprayed surfaces for control. Care should be taken to mark the squares at different heights on the walls. Bioassays should be carried out on the marked squares to assess the residual activity of the given insecticide. Inhabitants should be advised not to physically alter the marked areas, mud plaster, white wash/ paint, etc.

Box 2: State-wise spray schedule, as recommended by the NVBDCP

Spraying is usually started to coincide with the buildup of vector populations and before peak malaria transmission. The recommended State-wise spray schedule for DDT and synthetic pyrethroids is given below. Two rounds of sprays are done for DDT and synthetic pyrethroids to provide protection during the entire transmission season. Three rounds are required in case of malathion since the insecticide is effective over a shorter period. It is expected that the spray operations will start in time to cover the entire transmission season, which is usually about 5 to 6 months in most parts of the country.

| Month | Dates for I round spray (Date/Month)* | States |
|--------------------|--|---|
| March | 01/3 | Andaman & Nicobar Islands |
| | 15/3 | Karnataka, Meghalaya, Tripura, Mizoram, Arunachal Pradesh, Nagaland, Assam, Manipur. |
| April | 01/4 | Himachal Pradesh, Pondicherry |
| | 16/4 | Tamil Nadu |
| | 16/4 | Sikkim |
| | 16/4 | Punjab |
| May | 01/5 | Daman & Diu |
| | 01/5 | Andhra Pradesh, Bihar, Chandigarh, Chhattisgarh, Goa, Gujarat, Jammu & Kashmir, Jharkhand, Madhya Pradesh, Odisha, Uttaranchal Pradesh, Uttar Pradesh |
| | 15/5 | Haryana, Dadra & Nagar Haveli |
| | 15/5 | Rajasthan, West Bengal |
| June | 01/6 | Maharashtra |
| Focal Spray | | Delhi, Kerala, Lakshadweep |

Dates for subsequent rounds (II/ III) should be finalized on the basis of residual activity of the insecticide in weeks and in consultation with local health programme personnel/ NVBDCP.

Box 3: General specifications of spray pump and spraying (NVBDCP)

- Pump: Stirrup pump or hand compression sprayer (ISI mark).
- Nozzle tip: stainless steel flat-fan type; discharge rate of 740–850 ml per minute [If more than 850 ml, nozzle tip should be replaced].
- Distance and angle of lance from wall: 45 cm and 60°.
- Swath width: 53 cm (21") with 3 inches overlap while spraying.
- Operation of stirrup pump plunger: 20–26 strokes per minute; 10–15 cm movement.
- 10 psi pressure at the nozzle tip
- Operation of hand compression pump: Above discharge rate should be attained at 40 psi
- Rate of coverage: 5 min per house with an average sprayable surface of 150 m²

Box 4: Norms of spray of NVBDCP (Source: MAP 1995)

- To cover 1 million population, 52 squads of 5 members each are required for 5 months.
- Squad comprises 2 pump men; 2 spray men; 1 insecticide suspension supplier and a superior field worker to supervise and keep record.
- Every squad should cover 60–80 houses in plain area and 50–60 houses in hilly/foothill area each day.
- Each squad receives 2—Pumps; 2—Nozzle tips, 4—15 litre bucket; 1— 5/10litre bucket; 3 m—Asbestos thread; 1— Measuring mug (500 g); 1m²— Straining cloth; 2—Pump washers; 3 x 3m—Plastic sheet.

Table 4. Requirement of insecticide per house (average sprayable area 50 m²)

| % formulation (a.i.) | Dosage (a.i.) /m ² | Insecticide | Insecticide for 10 litres |
|----------------------|-------------------------------|-------------|---------------------------|
| 50% | 1 g | 300 g | 1000 g |
| 25% | 1 g | 600 g | 2000 g |
| 10% | 25 mg | 37.5 g | 125 g |
| 5% | 25 mg | 75 g | 250 g |
| 2.5% | 25 mg | 150 g | 500g |

*10 litres of suspension should be sufficient for 3 rural houses with sprayable surface area of ~ 500 m².

Table 5. Details of spray and insecticide consumption

Name of the villagePHCDistrict

Statepray Squad No. Name of the Sr.F.W./Supervisor

Insecticide and formulation..... Date of spray

Spray round.....

| S. No | H.No. | HOF | Houses | | Rooms | | %HC | %RC | Insecticide consumed in kg |
|-------|-------|-----|---------|-----|----------|---------|-----|-----|----------------------------|
| | | | Sprayed | L R | Targeted | Sprayed | CS | TS | |
| 1. | | | | | | | | | |
| 2. | | | | | | | | | |
| Total | | | | | | | | | |

HOF: Head of family; L: Locked; R: Refused; CS: Cattle shed; TS: Temporary shed; % coverage = Number sprayed x 100/Number targeted; HC: House coverage; RC: Room coverage

Box 5: Safety measures (Source: Malaria Vector Control WHO/WHOPES/2002.5 and Environmental Codes of Practice (ECoP) - NVBDCP (<http://nvbdcp.gov.in/ECoP.html>))

Spray-men

- All spray personnel should wear appropriate personnel protective equipments (PPE) during the entire operation, such as goggles, gloves, boots and two sets of working clothes
- Spray men should wash hands and face every time after insecticide is handled.
- Eating, drinking and smoking should be avoided while spraying.
- After completion of spraying, the spray-men should remove the PPE and wear fresh sets of clothes before eating or drinking
- Spray-men should be advised to take bath after each day’s work.
- Spray-men should ideally work for 5-6 hours a day.
- If there is skin-contact with insecticides, the affected area should be immediately washed off with soap and water. If insecticide goes into the eyes, they should be immediately flushed out with plenty of water.
- In case of poisoning by insecticides, the following antidotes should be used:

In case of organophosphate (targets nervous system and is an esterase inhibitor) poisoning, 2-4 mg of atropine should be given intravenously (for children 0.5 to 2 mg according to weight). Depending on symptoms, further doses of 2 mg should be given every 15 minutes for 2-12 hours in severe cases.

Box 5: continued

Synthetic Pyrethroids poisoning (affects every part of the nervous system)

- Vitamin E oil preparations can be given for prolonged paraesthesia. Only in cases of definite allergic symptoms, should corticosteroids be administered. On occurrence of convulsions after severe intoxication, intravenous injection of 5-10 mg Diazepam (or other benzodiazepine derivatives) should be given.

Inhabitants

- Food, cooking utensils, bedding, clothing, portable furniture in the house should be removed from the house before spraying; children and sick people should be temporarily shifted.
- Spray-men should remove, or assist the house owner in removing, all calendars, papers, photo frames that are placed on the walls, before starting spraying operation.
- Inhabitants should not enter the sprayed rooms until the spray is dry, and instructed to sweep the floors before allowing small children or indoor domestic animals into the rooms
- Immovable property or furniture which cannot be removed should be gathered in the middle of the room and covered with polythene sheet. Heavy furniture that cannot be moved or household items which cannot be taken out of the house should also be covered
- A double coloured polythene sheet should be used for covering the furniture. Double coloured (blue and red or yellow and blue) polythene sheets or single coloured sheets duly marked (with cross signs) should be spread on the stockpile before spraying. The red or yellow or marked side should be the bottom surface, i.e. the surface facing the stockpile or the furniture
- In case there is any stockpile of food grain in the house, it should be removed if possible or covered before spraying is carried out.
- Any adverse effect of spray to inhabitants and spray men or accidental exposure to insecticides should be informed to the supervisor. Supervisory staff should know the first aid procedures for insecticides exposures and information on the nearest medical facility. The patient should be immediately moved to well ventilated area, contaminated clothes removed, clothes loosened and taken to medical facility. Head should always be kept upright not to obstruct respiration while transportation. Supervisor should provide the label of the insecticide container to the medical officer for advice on antidote. Supervisors should be trained for giving resuscitation (artificial respiration).

Box 6: Supervision of spraying

Concurrent

- Date of spray, advance notification, scheduling, spray crew
- Discharge rates of nozzles, condition of pumps
- Preparation of suspension and supervision for technique, speed, coverage, safety, etc.
- Squad supervisors should ensure that the spray-men never work alone while spraying.

Consecutive

- Evidence of spray deposits
- Uniformity of spray
- Coverage, if less, reasons (refusals or locked premises or others)
- Mopping up of operations to spray unsprayed houses
- Extent of defacing of spray by mud-plastering, white wash, etc. and reasons.

The bioassays should be done on day 1 and day 7 post-spraying and thereafter fortnightly/ monthly using WHO cones. Batches of 10 non-blood-fed mosquitoes, 2–5 days old, are released in to each cone and exposed for 30 minutes on each of the walls of each hut. Wherever, it is not practical to use non-blood fed 2-5 days old (F1 females) mosquitoes for the assay, wild caught blood-fed female mosquitoes may be used for cone bioassay. After exposure the mosquitoes are carefully removed and placed in plastic containers covered with nylon net fastened with rubber band. Mosquitoes are provided with 10% sucrose solution soaked in cotton wool. After 24 h of holding, percent mortalities are computed from the total number of alive and dead mosquitoes in the replicates for each type of surface and recorded in the format as given in Table 6.

The number of weeks/ months during which the mortality is above the “cut-off level” (at least 80% mortality after 24 hours’ holding) is recorded. After each exposure the kit should be washed with soap and clean water, and dried for next use. Results are expressed as residual activity against a given dosage of the insecticide.

Table 6. Cone bioassays for residual activity

Village Sub-centre..... PHCDistrict Date of bioassay.....
 Insecticide & Dosage Temperature: Exposure..... Holding..... Humidity.....
 Date of last spray and round.....Type of surface: Mud/cement/brick/thatch.....
 Test species..... Lab/F1/Field collected.....

| Replicates | House code | 30 minute knock-down (no.) | 24 h % mortality | Corrected % mortality | Remarks |
|-------------|------------|----------------------------|------------------|-----------------------|---------|
| Replicate 1 | | | | | |
| Replicate 2 | | | | | |
| Replicate 3 | | | | | |
| Replicate 4 | | | | | |
| Control 1 | | | | | |
| Control 2 | | | | | |

2.1.2.2.7. Entomological evaluation

The entomological collections should be carried out on regular intervals, preferably on day 1 and day 7 post-spraying and thereafter fortnightly/ monthly, concurrently with the assays conducted for residual activity. Evaluation for all indicators should be done in all sprayed and control houses on the same day. Data of each of the evaluation indicators should be entered in the respective proforma. The methods given below are for the evaluation of three dosages of a given insecticide (four replicates for each dosage) and equal number of controls. In case of comparison with another insecticide (positive control), additional houses should be selected.

2.1.2.2.7.1. Floor sheet collection

White cloth is spread on the preceding night on the entire floor of the house before inhabitants retire to bed. Next morning, before the inhabitants resume their regular activities, the dead and morbid mosquitoes lying on the floor sheet should be picked up with forceps and scored. Other dead insects lying on the floor should be separately collected and stored for monitoring. The inhabitants of the trial houses are asked not to physically damage the knocked-down mosquitoes. Precautions should be taken to protect the knocked-down mosquitoes from scavengers such as ants. These mosquitoes should be identified to species and abdominal (gonotrophic) condition recorded in the format given in Table 7. The floor sheet collections provide comparative data on immediate mortality after contact with insecticide sprayed surfaces at different dosages tested.

2.1.2.2.7.2. Mosquito collection indoors

Following the floor sheet collection, resting mosquitoes (indoor) in the house are collected using aspirator. Care should be taken to collect all resting mosquitoes to the possible extent.

The collections of mosquitoes are labelled according to the test arm and kept in 150 ml/ 300 ml cups (10 individuals per cup), with 10% sucrose solution and maintained in a climatic chamber for 24 hours at 27°C ± 2°C and 80% ± 10% RH. The percent mortality after 24 hours is recorded. Observation on number of dead mosquitoes after 24 hours of holding provides data on delayed mortality at different dosages tested (Table 8).

Table 7. Floor sheet collection

Village..... Sub-centre..... PHC District..... State.....
 Date of collection.....Insecticide & Dose.....Spray Round.....Type of structure:
 Mud/cement/brick/Thatch/
 stone/.....Temperature: Min.....Max..... Relative humidity: Min Max.....

| Arm No. (House code and dosage)* | Species | Males | Females | | | | Total no. (dead + morbid) |
|--|---------|-------|---------|----|----|---|---------------------------------|
| | | | UF** | BF | SG | G | |
| Dosage 1 | | | | | | | |
| Dosage 2 | | | | | | | |
| Dosage 3 | | | | | | | |
| Control 1 | | | | | | | |
| Control 2 (positive) | | | | | | | |

UF = Unfed; BF = Blood fed; SG = Semi-gravid; G = Gravid; *Separate row for each replicate;
 **: Unfed mosquitoes should be dissected for parity (Refer section 2.1.2.2.7.4.).

2.1.2.2.7.3. Exit window trap collection

Exit window traps should be fixed in all the houses in the evening hours before sunset in all the houses. Next morning all the mosquitoes from the exit traps should be collected. The dead mosquitoes should be placed in petridishes lined with moist filter paper for species identification and scoring. The live mosquitoes should be transferred to a cage and brought to the laboratory wrapped in a wet towel. The cage should be kept preferably in an unsprayed room. During the holding period optimum conditions for survival should be provided, i.e. 27± 2°C temperatures, 80% ± 10% RH and a glucose source. Where it is not possible to maintain temperature and relative humidity a climate chamber can be used to simulate the ideal condition. The mortality is scored after 24 hours holding period. The mosquitoes are identified to species and their abdominal (gonotrophic) conditions recorded in the format given in Table 9. This indicator provides data on relative excito-repellency property of the insecticide at different dosages tested.

Table 8. Hand catch collection

Village.....Sub-centre PHC District State
 Date of collection.....Insecticide & Dose..... Spray Round.....Type of structure:
 Mud/cement/brick/stone/.....Temperature: Min Max..... Relative humidity: Min..... Max

| Arm No. (House code & dose) | Species | Male | Female | | | | Total | Mortality after 24 h |
|-----------------------------------|---------|------|--------|----|----|---|-------|-------------------------|
| | | | UF* | BF | SG | G | | |
| Dosage 1 | | | | | | | | |
| Dosage 2 | | | | | | | | |
| Dosage 3 | | | | | | | | |
| Control 1 | | | | | | | | |
| Control 2 | | | | | | | | |

UF=Unfed; BF= Blood fed; SG= Semi-gravid; G= Gravid; * Unfed mosquitoes should be dissected for parity (Refer section 1.1.1.5g5). MHD– No. of mosquitoes collected/No. of persons X time (h).

Table 9. Exit trap collection

Village Sub-centre PHC District State.....
 Date of collection.....Insecticide & Dose..... Spray Round.....Type of structure:
 Mud/cement/brick/
 stone/.....Temperature: Min Max..... Relative humidity: MinMax.....

| (a) Dead mosquitoes | | | | | | | | |
|------------------------------------|---------|------|--------|----|----|---|-------|-------------------------|
| Arm No. (House code & Dose)* | Species | Male | Female | | | | Total | Mortality after 24 h |
| | | | UF** | BF | SG | G | | |
| Dosage 1 | | | | | | | | |
| Dosage 2 | | | | | | | | |
| Dosage 3 | | | | | | | | |
| Control | | | | | | | | |

| (b) Alive mosquitoes | | | | | | | | |
|------------------------------------|---------|------|--------|----|----|---|-------|-------------------------|
| Arm No. (House code & Dose)* | Species | Male | Female | | | | Total | Mortality after 24 h |
| | | | UF** | BF | SG | G | | |
| Dosage 1 | | | | | | | | |
| Dosage 2 | | | | | | | | |
| Dosage 3 | | | | | | | | |
| Control | | | | | | | | |

UF=Unfed; BF= Blood fed; SG= Semi-gravid; G= Gravid; * Separate row for each replicate; **Unfed mosquitoes should be dissected for parity (Refer section 1.1.1.5g5).

2.1.2.2.7.4. Parity

All unfed mosquitoes collected by different methods should be dissected and tracheolar skeins are observed to assess parity. In nulliparous mosquitoes the tracheolar skeins will be in coiled condition and in parous mosquitoes the skeins are distended (WHO 1975). Mosquitoes should be categorized as nulliparous and parous mosquitoes and recorded in the format given in Table 10. Reduced parity rate of the mosquito population indicates the reduction of their longevity. The data should be recorded for each dosage and control separately.

Table 10. Parity rates

Village Sub-centre PHC..... District..... State.....
 Insecticide & Dose.....Spray Round.....

| Species | Total dissected | No. nulliparous | No. parous | Percent parous |
|--|-----------------|-----------------|------------|----------------|
| 1. | | | | |
| 2. | | | | |
| 3. | | | | |
| Parity rate = Number parous / Total number dissected x 100 | | | | |

2.1.2.2.7.5. Airborne toxicity (cage method)

Some insecticides may produce air-borne toxicity. This can be assessed by estimating, in comparison with an unsprayed hut (control), the mortality of mosquitoes placed in small cages (5 to 10 cages in each hut, 25 non blood-fed female mosquitoes in each cage) hanging from the ceiling, for 4–8 hours up to a maximum of 12 hours, at different distances from the sprayed surfaces. The mosquitoes are then kept for 24 hours’ observation (at 27+2oC and 60–70% RH) after being transferred to clean cages. The mortality in comparison to the controls is recorded (as shown in Table 11) to assess the air-borne toxicity due to volatility of the insecticide.

Table 11. Airborne toxicity

Village Sub centrePHC District State.....
 Date of bioassay Household No Insecticide and dosage.....
 Spray Round.....Temperature: Min..... Max..... Relative humidity:
 MinMax.....Test speciesLab/F1/Field collected
 mosquito.....

| Replicate * Cage No. | No. of mosquitoes | No. knocked down after 1 h | No. killed after 24 h holding | % mortality after 1h | % mortality after 24h |
|-------------------------|----------------------|-------------------------------|-------------------------------------|-------------------------|--------------------------|
| Replicate 1 | | | | | |
| Replicate 2 | | | | | |
| Control | | | | | |

2.1.2.2.7.6. Significance of entomological indicators

- Floor sheet collections: Immediate mortality
- Aspirator collection indoors: Delayed mortality
- Exit trap collections: Induced exophily/ excito-repellency
- Dissection for parous condition: Reduction of parity rate indicates reduction of survival rate
- Air-borne toxicity (of volatile insecticides): Volatile effect

2.1.2.2.7.7. Assessment of impact of the insecticide on vector

| | |
|--|-----|
| Number of vector mosquitoes found dead on floor sheet in the morning | (a) |
| Number found dead in exit traps | (b) |
| Number found alive in exit traps | (c) |
| Number found dead after 24 h holding of live mosquitoes | (d) |
| Total entry = a+b+c+d | (e) |

- Comparisons between various treatments and control are made and the inferences drawn as under:
- Immediate mortality= $[a+b/e] \times 100$
- Excito-repellency= Comparative exit to entry rate $(b+c/e) \times 100$
- Delayed mortality= Number dead after 24 h among the live mosquitoes $[d/e] \times 100$
- Overall mortality= $[a+b+d/ e] \times 100$

2.1.2.2.8. Perceived side effects, acceptability by householders and collateral benefits

Information on these aspects will be collected by interviewing the inhabitants using structured questionnaire (Annexure 2).

2.1.2.2.9. Human safety

Collection of data on human safety should be accomplished by interviewing using structured questionnaire or by physically examining the inhabitants of both sprayed and unsprayed houses before and after spraying to record any change in certain health parameters due to insecticidal spraying (Annexure 3). A medical practitioner should be associated for collection of such data.

2.1.2.2.10. Criteria for selection of dosage of insecticide for Phase III trial

The dosage of the insecticide that gives the maximum reduction of human-mosquito contact; with >6 weeks residual activity and with minimum adverse effects should be the optimum application dosage to be selected for the Phase III trial.

2.1.3. Large-scale field trials (Phase III)

Duration: 12 months

The Phase III trials are carried out at village level selecting one or more villages. The efficacy of the insecticide formulations that are found suitable for IRS in experimental hut or small-scale field trials (Phase II) should be evaluated in large-scale (at village level) against the target mosquito populations at least in three eco-epidemiological settings (multi-centric).

Objectives

- To establish the efficacy of insecticide formulations at the selected dosage against the target vector species, when sprayed all or most households in the community;
- To confirm residual activity and application intervals
- To study the impact on disease incidence/ prevalence
- To assess community acceptability of the new insecticides or formulations and collateral benefits
- To observe ease of application and handling of the insecticide product, and to record perceived side-effects, if any, by operators and inhabitants of the sprayed houses.

2.1.3.1. Selection of villages and collection of baseline data

The Phase III trials are usually designed as cluster randomized trials and the unit of intervention under this phase is the village. The stated effect of insecticide used for IRS is to reduce the longevity, density and infectivity rate of the target vectors.

Selection of villages should be done in consultation with the state/ district health programme personnel. The villages selected for spraying and controls (unsprayed) should be eco-epidemiologically homogenous. Villages with an average annual parasite incidence (API) of ≥ 2 (in last 3–5 years) with a population of ~3000 (one or cluster of villages) should be selected for treatment (spraying). If one village is selected with ~3000 population, the village can be divided in to minimum three segments and each segment is considered as a replicate. For comparison (unsprayed controls), village(s) with a similar population size located at a distance of about 5–10 km from the treatment village(s) should be selected. The distance is maintained to avoid infiltration of mosquitoes in to the sprayed area. If known, the flight range of the vector species should be taken in to account while selecting the treatment and control villages. Where selection of well separated treatment and control villages is not feasible, the size of the area may be increased to include several villages for spraying and use its central part for evaluation, thus achieving a barrier between the sprayed and unsprayed villages. Such a barrier should be wider than the known or expected flight range of the vector. For multi-centric evaluation, the villages selected should be from different ecotypes (at least three) having different vector species and all the seasons prevailing in a year are to be covered for evaluation.

The selected villages should be randomly allotted to treatment and control arms in order to reduce the selection bias and to reliably assess the effect of indoor residual spraying with the given insecticide. If villages are heterogeneous, it is desirable to stratify them in terms of size, location (ecotype), vector density, types of breeding habitats, incidence of disease and use of

personal protection measures. The required baseline data for such stratification should be collected prior to the trial and comparable treatment and control groups selected. This process would take a few months to a year, depending on the entomological and transmission patterns of the area. Following the stratification, within each identified stratum, villages are randomly allocated to the treatment or control arms.

Also, the villages can be stratified in pairs (Matched pair designs) and from each pair one village is randomly assigned to the treatment arm and the other to the control arm. Stratified designs are usually preferable to matched pair designs. Cluster-randomized trials with lesser than five clusters per arm are not advisable, because the results obtained with parametric tests may be unreliable with smaller sample sizes. The number of entomological monitoring sites should be equal in each village. Since houses may vary greatly in their attractiveness to mosquitoes, for practical reasons and consistency, the same entomological sites should be monitored throughout the study (WHO/CDS/NTD/WHOPES/ GCDPP/ 2006.3).

Conducting IRS trials with negative controls is not acceptable for ethical reasons. A positive control, spraying of known insecticides such as DDT or deltamethrin, would be an acceptable alternative, but sometimes it may not be possible to show a difference in efficacy between the treatment and positive control arms. Therefore, as an alternative to a positive control, and to ensure an equivalent level of protection, early detection and prompt treatment of infections through intensified surveillance could be used.

2.1.3.2. Census

In collaboration with the respective Primary Health Centre and District Public Health department, census and numbering of all houses in the selected experimental and control villages should be carried out prior to spraying. Census details are recorded in the format as given in Table 12.

Table 12. Record of census of households

Village Sub-centre PHC..... District.....

State..... Date of survey

| S. No. | House No. | Name | Relation | Age/ Gender | Educa- tion | Profe- ssion | Type of Structure | No. of rooms | No. of cattle-sheds | No. of tempo-rary sheds | Sleeping habit inside/ outside |
|--------|-----------|------|----------|-------------|-------------|--------------|-------------------|--------------|---------------------|-------------------------|--------------------------------|
| 1. | | | | | | | | | | | |
| 2. | | | | | | | | | | | |
| 3. | | | | | | | | | | | |
| 4. | | | | | | | | | | | |
| 5. | | | | | | | | | | | |
| 6. | | | | | | | | | | | |

2.1.3.3. Ethical considerations

For conducting the trial at village level, necessary ethical clearance should be obtained from the ethical committees of the respective institutions/ authorities. The informed consent form and the information sheet containing the details of the trials to be provided to the villagers/ households should also get approved by the ethics committee.

The participants should be explained clearly in the vernacular/ local language about the objectives of the trial, study protocol, advantages to the people and inconveniences, if any expected. Participants should be told they have every liberty to participate or refuse to participate. The trial's information sheet, as approved by the ethics committee, should be made available to all participating villager.

2.1.3.4. Spraying of villages

In the treatment villages, the given insecticide or insecticide formulation is sprayed while in the control villages the insecticide in use in the programme can be sprayed or an equivalent forms of protection that has no effect on vector populations (e.g. chemoprophylaxis/ early diagnosis and prompt treatment, EDPT as per the national guidelines) should be used (refer to section 2.1.3.1.). Spraying in the treatment villages is done in collaboration with the respective PHC Medical Officer and District Malaria Officer. All human dwellings, mixed dwellings, cattle sheds, temporary sheds and other structures, if any, should be sprayed to ensure a total coverage. Similarly, to achieve a complete spraying, all sprayable surfaces (except floor) of the dwellings are sprayed with an uniform application of the target dosage. In case, the duration of the trial is longer than the duration of residual activity of the insecticide treatment, the spraying should be repeated.

Spraying should be done with the technique, equipments in use in the routine vector control programme under strict supervision following the NVBDCP guidelines. Inhabitants of the selected villages should be informed in advance for preparation to get their dwellings sprayed on the scheduled date and the benefits they are expected to get out of the spraying. Necessary precautions should be taken for the protection of spray men by providing personal protection equipment (refer Box 5). Each house is marked on the wall indicating date and coverage of spray [Number of rooms sprayed/ number of rooms in the dwelling (e.g. Dwelling No. 5/5)]. Coverage of spray should be recorded and presented as indicated in the Phase II (refer to section 2.1.2.2.4.).

During the entire period of the trial, a physician, who has experience in recognizing clinically the signs and symptoms of different types of insecticides poisoning should monitor the persons involved in the trial and respond to any adverse effect on health.. Parents of the households in the sprayed villages should be cautioned about the risky situations involving children.

2.1.3.5. Assessment of spray quality

To achieve a quality spray (uniform application with adequate dosage), it is essential that spray-men are properly trained and use spray equipments that are well-maintained and calibrated and ultimately the spray operation is closely supervised. Whatman® No. 1 papers are fixed to the walls of the houses selected randomly (3 sampling spots, 1 from roof and 2

from walls, in each of the minimum 10 houses selected per segment/ village) and removed after the spray operation and assayed for insecticide content. For chemical analysis, filter-paper samples are preferred to the scrapings of sprayed mud surfaces because of the difficulties in the process of standardization.

2.1.3.6. Assay for residual activity

Residual activity should be assessed as per the procedure described in section 2.1.2.2.6.

2.1.3.7. Evaluation

Evaluation should be done in the sprayed and control villages at fortnightly/ monthly interval in randomly selected dwellings representing different types (cemented, tiled, thatched, mud, etc.) and structures (human dwellings, mixed dwellings (if present) and cattle sheds).

2.1.3.7.1. Efficacy

Certain entomological parameters are relevant or required to assess the effectiveness of the insecticide spraying.

2.1.3.7.1.1. Vector density

Density of the vector species is measured using different methods, each with advantages and limitations.

Indoor resting density: Hand catches of resting mosquitoes indoors in the dawn hours are one of the reliable and practical methods of assessing the population density of the vector species and also facilitate estimating biting rates in areas where the vectors are zoophagic and where only small numbers of mosquitoes are obtained per night from human landing catches (HLC). The collections are identified to species and the gonotrophic condition of the female mosquitoes is recorded. In indoor resting catches, if the proportion of half-gravid or gravid mosquitoes is found reduced, it may be an indication of mortality induced by the insecticide or repellency. Indoor resting collections are also indicative of mosquito biting rates on human if the proportion of mosquitoes feeding on humans is known (WHO, 2006). The source of blood-meal of individual mosquitoes is identified using precipitin (agar-gel diffusion method) or ELISA tests. Using the product of indoor resting collection and the proportion that fed on man (human blood index, HBI), mosquito biting rate on human may be estimated. Hand catches of indoor resting mosquitoes in four to six houses per village at fortnightly/ monthly intervals will give meaningful data on mosquito density, which is expressed as the number per man-hour (man-hour density, MHD).

In addition, data on exit rate or repellent effect of the insecticide could be collected by fixing exit traps to the existing windows of the sprayed and unsprayed houses. In such case, the number collected from the exit traps is added to the hand catches and the density is expressed as the number of vectors captured per room per unit time,

Outdoor resting collections: Outdoor resting mosquitoes could be collected from the natural resting sites such as pit shelters, vegetation, root interstices, tree hollows available in and around the villages. However, searching natural shelters may not be feasible considering the

vastness of the area outdoors. Therefore, alternatively, artificial shelters, particularly those which resemble the natural ones and are attractive to the vector species for resting, could be installed and used for the collections (for example, pit traps (pit shelters) dug in the ground). Such collections may provide information on outdoor resting behaviour if the vector commonly rests outdoors or is driven outdoors by the repellent effect of the insecticide. Four to six shelters may be installed per village. The shelters should preferably be installed under shade and in such a way that they do not face the direction of sunrise. Mosquito collections are carried out in the morning hours and the density is expressed as number collected per shelter or per man-hour.

Mosquito landing collections on human (HLC) (This may be done if feasible and on obtaining necessary clearance from human ethics committee): The density of the vector species can also be monitored by conducting HLC that gives the number of landing mosquitoes per person per night. All night (dusk to dawn) mosquito landing collections should be made in one house in each treatment and control village at fortnightly intervals. Collections are required both inside and outside the houses to assess biting rate indoors and outdoors (endo- and exophagy). Persons volunteering to be baits should be informed about the experiment. Informed consent of the volunteers involved in the study should be obtained prior to the collections (Annexure 4). The human volunteers may lie down or on a cot and can sleep as per their normal sleeping practice, exposing legs up to knees. The insect collectors, who will be catching the mosquitoes landing on the bait, are rotated every four hours to avoid bias and slackness. The sampling errors caused by variation in catcher efficiency or attractiveness may be reduced by increasing the number of capture sites per cluster. Hourly mosquito collections should be recorded in the format given in Table 13. Results are expressed as number of vectors landing per human bait per night. If the collections are restricted to the hours of peak biting of the vector species, the results are expressed as number of mosquitoes landing per bait per hour. The results would provide information on biting periodicity and feeding habits of the vector species in the study areas.

Mosquito landing collections on animals (This may be done if feasible and on obtaining necessary clearance from animal ethics committee): In areas, where the vectors are mainly zoophagic or present at low densities, HLC results in low capture rates and poor catcher efficiency. Therefore, to measure more accurately the abundance of the zoophagic vector(s) in a sprayed cluster, collections of landing mosquitoes on domestic animals (usually cattle) are made at fortnightly intervals. Landing collections on a cattle tied to a pole are made from dusk to dawn. Data should be recorded in the format given in Table 14. Results are expressed as number of vectors landing per animal bait per night or number of mosquitoes per bait per hour, if the collections are restricted to the hours of peak biting. This will provide information on biting rhythm and feeding habits of the vector species in the area.

Light trap catches: In areas, where there is a correlation between the light trap (hung at the side of occupied untreated nets) catches and HLC, light trap catches can replace HLC. Light trap Mosquito collection using light traps is relatively easier and much less labour-intensive than HLC. Therefore, light traps (CDC light traps or its modified versions) could be a reliable alternative that overcome the ethical constraints and remove human error (while mosquito collection) associated with HLC. The traps are set indoors (human dwellings or animal sheds)

as well as outdoors during dusk hours at fortnightly intervals in both treated and control villages. The next morning, the trapped mosquitoes are collected, identified to species and recorded in the format given in Table 15.

Table 13. Mosquito landing collection on human (HLC)

Village..... Sub-centre..... PHC.....District State.....

Date of collection.....Insecticide & Dose.....Spray Round.....Temperature:
Min.....

Max Relative humidity: Min Max

| Time (Hrs) | Malaria vector | | | | | Other anophelines | | <i>Cx. quinquefasciatus</i> | | Other culicines | |
|---|----------------|-----|----|----|---|----------------------|---|-----------------------------|---|--------------------|---|
| | M | UF* | BF | SG | G | M | F | M | F | M | F |
| | T NP P | | | | | | | | | | |
| 1800 | | | | | | | | | | | |
| 1900 | | | | | | | | | | | |
| 2000 | | | | | | | | | | | |
| 2100 | | | | | | | | | | | |
| 2200 | | | | | | | | | | | |
| 2300 | | | | | | | | | | | |
| 2400 | | | | | | | | | | | |
| 0100 | | | | | | | | | | | |
| 0200 | | | | | | | | | | | |
| 0300 | | | | | | | | | | | |
| 0400 | | | | | | | | | | | |
| 0500 | | | | | | | | | | | |
| 0600 | | | | | | | | | | | |
| Total | | | | | | | | | | | |
| M = Males; UF = Unfed females; BF = Blood fed females; SG = Semi-gravid females; G = Gravid females; P = Parous; NP = Nulliparous; T = Total dissected; *Unfed mosquitoes should be dissected for parity (see section 2.1.2.2.7.4.. | | | | | | | | | | | |

Table 14. Mosquito landing collection on animal

Village..... Sub-centre.....PHC.....
 District..... State.....
 Date of collection.....Insecticide & Dose.....Spray Round.....Temperature:
 Min..... Max.....Relative humidity: Min
Max.....

| Time (Hrs) | <i>An. culicifacies</i> | | | | | Other anophelines | | <i>Cx. quinquefasciatus</i> | | Other culicines | |
|--|-------------------------|-----|----|----|---|-------------------|---|-----------------------------|---|-----------------|---|
| | M | UF* | BF | SG | G | M | F | M | F | M | F |
| | ----- | | | | | | | | | | |
| | T | NP | P | | | | | | | | |
| 1800 | | | | | | | | | | | |
| 1900 | | | | | | | | | | | |
| 2000 | | | | | | | | | | | |
| 2100 | | | | | | | | | | | |
| 2200 | | | | | | | | | | | |
| 2300 | | | | | | | | | | | |
| 2400 | | | | | | | | | | | |
| 0100 | | | | | | | | | | | |
| 0200 | | | | | | | | | | | |
| 0300 | | | | | | | | | | | |
| 0400 | | | | | | | | | | | |
| 0500 | | | | | | | | | | | |
| 0600 | | | | | | | | | | | |
| Total | | | | | | | | | | | |
| M= Males; UF= Unfed females ; BF= blood-fed females, SG= Semi-gravid females, G= Gravid females; P = Parous; NP= Nulliparous; T= Total dissected; * Unfed mosquitoes should be dissected for parity (see section 2.1.2.2.7.4.. | | | | | | | | | | | |

Table 15. Light trap collection

Village.....Date of collectionInsecticide &
 Dosage..... Spray Round.....Collection site No & Type.....
 Temperature: Min..... Max Relative humidity: Min Max

| Trap Collection No. | site No. and type | <i>An. culicifacies</i> | | | | | Other anophelines | | <i>Cx. quinquefasciatus</i> | | Other culicines | |
|---|-------------------|-------------------------|-----|----|----|---|-------------------|---|-----------------------------|---|-----------------|---|
| | | M | UF* | BF | SG | G | M | F | M | F | M | F |
| | | ----- | | | | | | | | | | |
| | | T | NP | P | | | | | | | | |
| 1 | | | | | | | | | | | | |
| 2 | | | | | | | | | | | | |
| 3 | | | | | | | | | | | | |
| M= Males; F= Females; UF= Unfed females ; BF= blood-fed females, SG= Semi-gravid females; G= Gravid females; P = Parous; NP= Nulliparous; T= Total dissected; * Unfed mosquitoes should be dissected for parity (see section 2.1.2.2.7.4.) | | | | | | | | | | | | |

2.1.3.7.1.2. Vector longevity

IRS is done primarily to reduce the longevity (survival) of vector mosquitoes and thereby the probability of transmitting disease (malaria). For estimation of mosquito longevity in the field, the simplest method is to calculate the proportion of parous females in the given mosquito samples obtained from HLC or hand catches. The ovaries of unfed or freshly fed female mosquitoes are dissected out to examine whether the tracheoles are coiled or uncoiled. Uncoiled tracheoles indicate that a female has developed and laid eggs at least once in her lifetime. The proportion of such parous females with uncoiled tracheoles is used to estimate (indirectly) the probability of daily survival of mosquitoes in the population. If IRS with a given insecticide is effective, a marked reduction of parous mosquitoes in the population should be observed.

2.1.3.7.1.3. Infection and infectivity rates

Vector mosquitoes obtained from HLC and indoor resting hand catches are dissected out in 0.6% saline to examine their mid-gut for the presence of oocysts and salivary glands for sporozoites using microscopy (WHO 1975) or ELISA (Wirtz et al., 1985 & 1992) or PCR (Vythilingam et al., 1999). If the IRS is effective, only a few mosquitoes would survive the time required for sporozoites to develop and mature, and therefore a marked reduction of the sporozoite rate is expected. In areas, where the infection/ infectivity rate is very low, pooled samples (pool size needs to be standardized) can be used for ELISA test with no loss of sensitivity. By testing pooled samples, the numbers of tested mosquitoes could be increased to thousands which is necessary to conclude that there is a significant reduction after IRS and also to make meaningful comparisons between study arms. Results are recorded in a format as given in Table 16. Data should be represented for each insecticide and dosage separately.

Table 16. Vector infection and infectivity rates

Village.....Sub-centre.....PHC.....District.....
 Date of collection Insecticide & Dose.....

| Species | Number dissected | Oocyst positive | Sporozoite positive | Oocyst rate | Sporozoite rate | ELISA positive | PCR positive |
|--|------------------|-----------------|---------------------|-------------|-----------------|----------------|--------------|
| 1. | | | | | | | |
| 2. | | | | | | | |
| 3. | | | | | | | |
| Oocyst/sporozoite rate = $\frac{\text{Number found with oocysts/sporozoites}}{\text{Total number dissected}} \times 100$ | | | | | | | |

2.1.3.7.1.4. Entomological inoculation rate (EIR)

This important entomological indicator is used to measure the impact of a vector control intervention on interruption of disease transmission besides assessing the relative role of the vector species in transmission of the disease. EIR is defined as the estimated number of infective bites received by a person per night through the vector population. It is the product of sporozoite rate and the human landing rate (number of mosquitoes per bait per night) An effective insecticide treatment should reduce both the components of EIR.

$$\text{EIR} = \text{Sporozoite rate (\%)} \times \text{No. of mosquitoes per bait per night in mosquito landing collection on human}$$

2.1.3.7.1.5. Disease prevalence

Point prevalence of disease (malaria) through sample blood surveys (covering a minimum 10% of the total population in each of the study arms) should be assessed in treatment and control areas. The frequency and time of the survey should be decided based on the transmission pattern of the disease and in consultation with state health department. It is recommended that in case of two rounds of spraying, 1st sample blood survey should be conducted 15 days prior to first round of spraying, 2nd survey 30 days after first round of spraying, 3rd survey 30 days and 4th survey 70–80 days after 2nd round of spraying. Surveys may be carried out following systematic sampling method selecting houses depending on the total number of households to be selected in each village, which will be proportionate to the population size (PPS) of the villages. Rapid diagnostic kits (RDKs)/ microscopic examination of stained blood smears may be used to screen the inhabitants of the selected houses for malaria infection. The test/ microscopic positive persons will be administered with anti-malaria drugs as per the national guidelines. The health workers of the respective PHC will be involved in the treatment of malaria positive cases. Sample survey data are recorded in the format given in Table 17 a & b. Further the data may be arranged according to the following age groups; 0–11 & 12–24 months, 2–4, 5–9, 10–14 years, and 15 years and above. The disease prevalence is expressed as slide positivity rate (SPR)

$$\text{SPR} = \frac{\text{Number of slides positive for malaria parasites}}{\text{Total slides collected and examined}} \times 100$$

2.1.3.7.1.6. Disease incidence

Fever surveillance should be carried out in the treated and control villages while visiting them for entomological collections to record incidence of malaria. People suffering from fever and/ or other malaria symptoms and also those suffered from fever and/ or other symptoms between the last and current visit will be screened at fortnightly interval for malaria parasite infection using bivalent rapid diagnostic kits. Blood smears will be collected from RDT-negative patients and screened microscopically for malaria infection other than Plasmodium falciparum and P. vivax. All malaria positive cases will be administered anti-malarial drugs following the NVBDCP Guidelines. Severe cases, if any, will be referred to the nearest CHC. The incidence of malaria is expressed as SPR or Incidence against 1000 population on monthly (monthly parasite incidence, MPI) or yearly basis (annual parasite incidence, API)

$$\text{MPI/ API} = \frac{\text{Number tested positive}}{\text{Total population}} \times 1000$$

Total population

Table 17. Mass blood survey

a. Data collection sheet

Village..... Sub-centre..... PHC..... District.....
 Date of collection

| S. No. | H. No. | HOF | Name of patient | Age | Sex | Blood smear No. | Fever history | Drugs given | Result of smear | | |
|--------|--------|-----|-----------------|-----|-----|-----------------|---------------|-------------|-----------------|----|-----|
| | | | | | | | | | Pv | Pf | Mix |
| 1. | | | | | | | | | | | |
| 2. | | | | | | | | | | | |
| 3. | | | | | | | | | | | |
| 4. | | | | | | | | | | | |

b. Summary Sheet

Village..... Sub-centre..... PHC..... District..... State.....
 Date of collection Insecticide & Dose..... Date of spray/round

Part 1

| S.No | Name of Village | Population | | | | | Total |
|------|-----------------|------------|-----|-----|------|-----|-------|
| | | <1 | 1-4 | 5-8 | 9-15 | >15 | |
| 1. | | | | | | | |
| 2. | | | | | | | |

Part 2

| Blood smears collected | | | | | Drugs consumed | | | | |
|------------------------|-----|-----|------|-----|----------------|------|-----|-------------|-------------|
| <1 | 1-4 | 5-8 | 9-15 | >15 | Total | 4 AQ | 8AQ | Paracetamol | Other drugs |
| | | | | | | | | | |

Part 3

| Positives detected | | | | | % blood smears collected | Positives | | | | Remarks |
|--------------------|-----|-----|------|-----|--------------------------|-----------|----|----|-------|---------|
| <1 | 1-4 | 5-8 | 9-15 | >15 | | Total | Pv | Pf | Mixed | |
| | | | | | | | | | | |

2.1.3.7.1.7. Significance of indicators

- **Hand catch:** Relative density of vector mosquitoes and non-target insects
- **Human/animal bait catches:** Feeding preference, biting rhythm and man-vector contact
- **Light trap collections:** Where there is a correlation between light trap catches (set beside occupied untreated nets) and HLC, light trap catches can replace HLC, and exophilic behaviour of the vector(s)
- **Parity:** Longevity/ survival of the vector species
- **Vector infection/ infectivity:** Intensity of transmission and role of different vectors
- **Entomological inoculation rate:** Transmission load (force of infection)
- **Sample blood survey: Disease prevalence** (total Parasite load in the community)
- **Active surveillance:** Disease incidence (occurrence of new cases in the community)

2.1.3.7.1.8. Adverse effects, acceptability by householders and collateral benefits

These aspects are assessed by interviewing the inhabitants of the experiment villages using structured questionnaire (Annexure 2).

2.1.3.7.1.9. Human safety

This is accomplished by interviewing the spray-men and other handlers of the insecticide using structured questionnaire (Annexure 3). A medical practitioner should be associated for collection of data.

2.1.3.7.1.10. Operational acceptability

- Ease of application
 - % Suspending ability of the wettable powder formulation should be within the limits mentioned in the technical data sheet
 - Difficulty in pumping, repeated clogging of nozzle, stability of suspension, maintenance need of equipment, nozzle corrosion, nozzle discharge rate etc. should be ascertained from spray-men and supervisory staff
 - Stability of insecticide suspension for sufficient time after mixing
 - Stability of insecticide formulations in different storage conditions
 - Safety to spray-men and inhabitants (as enquired and investigated in Annexure 3)
 - Acceptability by community as determined by odour, effect on décor of premises, collateral benefits etc. (as enquired in Annexure 2)

2.1.3.7.1.11. Data analysis and interpretation

For the Phase III evaluation, the primary unit of replication and analysis is the village. The statistical method to be used for the analysis should adjust the variation existing between

villages before estimating the effect of the insecticide spraying. Multivariate analysis is therefore the preferred approach since it takes in to account such variations. Data on proportions (e.g. parous rates, sporozoite rates, bioassay mortality) should be analyzed using logistic regression. Considering the possibility of over dispersion (i.e. not normally distributed between sites), the numeric entomological data (e.g. mosquito resting density, human landing catches or light trap catches) should be analyzed using Poisson regression or transformed using logs to a normal distribution before applying analysis of variance.

The entomological indicators when analyzed provide information on the impact of the insecticide spraying on malaria transmission as indicated by the estimates of EIR which is derived from these indicators. EIR, the product of sporozoite rate and mosquito landing rate on human (HLC) is increasingly being used to measure the impact of vector control interventions on disease transmission. An overall analysis of entomological indicators will provide estimates of the efficacy of the treatment, while an analysis done by period may show changes in residual impact of the intervention over time (WHO, 2006).

2.2. Insecticide Treated Nets & Fabrics

Use of insecticide treated nets (ITNs) is a preventative method of control of malaria and kala-azar as ITNs when used practically prevent mosquito bites. Mosquito nets are impregnated with insecticide that kill mosquitoes upon their contact with the net and its efficacy gets enhanced when its use coincides with the seasonal abundance and biting rhythm of the mosquitoes and the sleeping habit/ time of the people who use them. There are also collateral benefits to the users including personal protection from other haematophagous insects. This vector control tool is eco-friendly as it minimises consumption of insecticides in the control programme. Thus, the use of ITNs has become an important component in malaria control, and many countries have developed strategic plans to upscale ITNs' use. However, one of the operational challenges facing large scale implementation of ITNs programme is their re-treatment every six to 12 months with insecticides. Consequent to overall low retreatment rates of the bed nets, long-lasting insecticidal nets (LLINs) have been developed, which require no further treatment throughout their expected life span of about three years or even more, making them more convenient and preferred over the conventional ones.

Presently, in endemic states of India, NVBDCP distributes LLINs, which are granted with full recommendation by the WHO, for malaria control. In addition, the health departments organize campaigns for impregnating community owned nets using the recommended synthetic pyrethroids at the recommended dosages. For impregnation of nets with the WHOPES passed insecticides, evaluation should be carried out at two Phases, Phase II and Phase III, whereas for new insecticides, three phases, Phase I, II and III, of evaluation are required.

2.2.1. Nets requiring treatment (WHOPES passed insecticides)

2.2.1.1. Phase II evaluation (Field study with village huts)

Duration: 12 months

Objectives

- To determine the efficacy of the selected insecticide on a given fabric or different fabrics against the target mosquito species (operational)
- To determine the optimum application dosage of insecticide for Phase III evaluation (technical) and its persistence
- To assess the safety aspects of impregnation and use (managerial)

2.2.1.1.1. Selection of dosages for treatment

For conventional treatment of mosquito nets (to prepare ITNs), WHO recommended insecticides (www.who.int/whopes/quality/en/) can be used and minimum three dosages may be selected.

2.2.1.1.2. Selection of study area

Study area should be selected in consultation with respective State/District health department to ensure that the area is free of ITN/LLIN use and does not receive other vector control

measures also. If the study is to be carried out in two or more villages, care should be taken to select eco-epidemiologically homogenous villages. Villages, potential for collection of adequate number of the target vector mosquitoes need to be selected for the trial. This could be ensured through base-line studies. Simultaneously studies should be undertaken in selected villages to examine biting rhythm of the vector species by mosquito landing collections (refer section 2.1.3.7.1.1.), resting behaviour of the vector species by light trap collections (refer section 2.1.3.7.1.1.) and susceptibility of the prevailing vectors to different insecticides (refer section 2.1.1.2, Box 1).

2.2.1.1.3. Selection of houses

In the selected village(s) 20 houses are chosen. These houses should be designated for interventions among four arms. Each house should be labelled with the number of the arm and the dosage of the insecticide. The houses in each arm should be distributed equitably among the dosages of impregnation and control - one house for each dosage and one for control as shown in Table 18.

In case of a request for comparison with another insecticide for impregnation, additional five houses should be selected (total 25 houses) and distributed as shown in Table 19. The selection of comparison-insecticide with the dosage of impregnation should be made in consultation with the NVBDCP, Delhi. Selection of houses should be with the written consent (Annexure 5) of the head of the family with permission to alter the structure of the house with minor changes, if needed.

Table 18. Distribution of houses in different arms

| Replicates | Houses with nets impregnated with candidate insecticide | | | Houses for negative control |
|------------|---|----------|----------|-----------------------------|
| | Arms | | | |
| | Dosage 1 | Dosage 2 | Dosage 3 | |
| 1 | House-1 | House-2 | House-3 | House-4 |
| 2 | House-5 | House-6 | House-7 | House-8 |
| 3 | House-9 | House-10 | House-11 | House-12 |
| 4 | House-13 | House-14 | House-15 | House-16 |
| 5 | House-17 | House-18 | House-19 | House-20 |

Table 19. Distribution of houses in different arms along with comparison insecticide as positive control

| Arm | Houses with nets impregnated with candidate insecticide | | | Houses for negative control | Houses for positive control |
|-----|---|----------|----------|-----------------------------|-----------------------------|
| | Dosage 1 | Dosage 2 | Dosage 3 | | |
| 1 | House-1 | House-2 | House-3 | House-4 | House-5 |
| 2 | House-6 | House-7 | House-8 | House-9 | House-10 |
| 3 | House-11 | House-12 | House-13 | House-14 | House-15 |
| 4 | House-16 | House-17 | House-18 | House-19 | House-20 |
| 5 | House-21 | House-22 | House-23 | House-24 | House-25 |

In case the ongoing intervention is suspended, the surveillance mechanism should be strengthened for providing protection from the disease to the villagers. Where feasible, hamlets or small villages having the required number of houses can be selected for the evaluation.

2.2.1.1.4. Treatment and distribution of nets

The exact number and sizes of nets to be distributed among the households should be decided after a census of the study area (Table 20). Assessment of requirement of nets should be made to protect all the inhabitants of the selected houses. As recommended by the NVBDCP, for a family of five members, two nets are to be given (@ one net per 2.5 persons). The procedure for impregnation of nets is given in Box 7.

Inhabitants should be educated on the use and storage of nets and instructed not to wash the nets. All the nets should be marked with a water-soluble ink to detect whether the nets are washed or not. Every time the team goes for data collection the marking on the nets should be checked and recorded. To ensure the regular use of nets by the inhabitants, volunteers from the village should be involved to ascertain the compliance. Net use rate should be assessed fortnightly and the data should be recorded in the format given in Table 21.

Table 20. Record of census of households

Village..... Sub-centre..... PHC..... District..... State.....
House No. Type of the structure..... Date.....

| Sl.No. | Name | Age/ gender | Education | Profession | No. of rooms | Sleeping habit Inside/ outside | No. of nets required |
|--------|------|----------------|-----------|------------|-----------------|-----------------------------------|-------------------------|
| HOF | | | | | | | |
| 1 | | | | | | | |
| 2 | | | | | | | |
| 3 | | | | | | | |
| 4 | | | | | | | |

Box 7: Procedure for impregnation of nets and fabrics

Specification of mosquito nets would be polyester- polyamide nets with 75-100 denier* (weight in grams of 9,000 m yarn) is optimal , white or single colour, 156 mesh (range 120-200 mesh per sq. inch) (minimum acceptable- mesh size 1.2 mm X 1.2mm in malarious areas; 0.6 mm X 0.6 mm in kala-azar areas)(12 x 13 holes)/square inch. The dosages (mg/ sq m) of impregnation for the study would be as follows:—

- (1) Surface area of the net (m²) = 2 [(length x height) + (height x width)] + (length x width)
- (2) Volume of the water required for soaking one net = Initial volume of water – Volume of water remaining in the tub after complete soaking.
- (3) Active ingredient (a.i.) of insecticide required for treatment of a net (mg) = Surface area in square metres x Target dosage (mg/sq m)
- (4) Volume of formulation required for a net = Weight in mg x 100/ Concentration of the insecticide formulation.

The required volume of formulation should be added to cold water in a tub (15–20 litres capacity). Where feasible it is recommended that suspension required for 3 nets should be prepared and used for impregnation of two mosquito nets to ensure uniform impregnation. Repeated rubbing and squeezing should be carried out while the nets are in the tub (15 minutes). Remaining liquid in the tub should be discarded appropriately. The treated mosquito nets should be dried in shade on a plastic sheet. After drying, nets should be properly labelled with insecticide dosage, date of impregnation on the cloth skirting at the bottom of the net with permanent marker. The nets should be placed in polyethylene bags and stored at room temperature safely.

- Same procedure is applicable for impregnation of nets/curtains of different fabrics
- For tablet formulation follow the kit instruction given by the manufacturer

Table 21. Record of compliance of treated nets in households

Village.....Sub-centre.....PHC.....District.....State.....House No.....

Insecticide & Dose.....Type of the structureDate of survey

| House code (Arm No./ Insecticide and dosage) | Date and time of visit | Use of net | |
|---|------------------------|------------|----|
| | | Yes | No |
| 1. | | | |
| 2. | | | |
| 3. | | | |

2.2.1.1.5. Persistent activity studies

Persistence is assessed by cone bioassays and if the phase II trial is carried out in different ecological settings, persistence needs to be assessed for each area.

2.2.1.1.6. Cone bioassays

Persistence of insecticidal activity will be assessed by conducting WHO cone bioassays (WHO 1998). The cone is placed on the treated net and its rim is fastened to the net with a rubber band. The cones will be fixed randomly on different flaps of the net for each dosage. One net from each dosage and one from control should be collected randomly from the inhabitants. Preferably 2-5 day old non-blood fed laboratory strain of the vectors or F1 female progeny of field collected adults or females of mixed age (using females of same gonotrophic condition) populations from unsprayed villages can be used for bioassays. Five mosquitoes should be introduced into each cone through its orifice which is plugged with cotton wool/ a polypropylene plug. At least two replicates should be used on each of the five sections (roof and four sides) on each treated and control net (refer to section 2.2.3.2.2.6.). Thus, 10 replicates of 5 mosquitoes in each replicate are used for each test sample, giving a total of 50 mosquitoes per net sample. Number of mosquitoes knocked-down at the end of three minutes and at the end of one hour should be recorded and the mosquitoes are transferred to plastic containers with a nylon net fastened with rubber band. Mosquitoes are provided with sugar soaked cotton wool placed on the net. The plastic containers should preferably be placed in an unsprayed room maintained at standard temperature ($27 \pm 2^\circ\text{C}$) and relative humidity ($80\% \pm 10\%$); where it is not feasible to maintain such conditions, a moist chamber can be used as described by WHO (1981). Percent mortalities are calculated after 24 h of holding from the alive and dead mosquitoes and corrected to the control mortality, if the control mortality is between 5 and 20%, using Abbott's formula. Data should be recorded in the format given in Table 22. Results are expressed as overall persistence effect of treated net at a given dosage of insecticide.

Table 22. Cone bioassay

Village..... Sub-centre..... PHC..... District.....State.....
 House No. Insecticide and dose.....Type of the structure.....Date of survey.....
 Test species..... Lab/ F₁/Field collected.....

| Net details | No. exposed | Number knocked-down after 3 minutes | No. dead after 24 h | % Mortality | Corrected % mortality* | (#10) |
|---|-------------|-------------------------------------|---------------------|-------------|------------------------|-------|
| Replicate 1 | | | | | | |
| Replicate 2 | | | | | | |
| Replicate 3 - 10 | | | | | | |
| Control 1 | | | | | | |
| Control 2 | | | | | | |
| Control 3-5 | | | | | | |
| % Mortality in test replicates – % Mortality in control *Corrected % mortality = -----x 100 100 – % Control mortality | | | | | | |

2.2.1.1.7. Entomological evaluation

Total number of mosquitoes in a structure should be measured every fortnight by the following methods in sequence as mentioned below in sections 2.2.1.1.8a to 2.2.1.1.8c.

2.2.1.1.7a. Floor sheet collection

For details see section 2.1.2.2.7.1.

2.2.1.1.7b. Exit window trap collection

For details see section 2.1.2.2.7.3.

2.2.1.1.7c. Hand catch

For details see section 2.1.2.2.7.2.

2.2.1.1.8. Significance of entomological indicators

Floor sheet collections: Immediate mortality

Exit trap collections: Excito-repellency

Hand catch: Relative density of mosquitoes and other non-target insects

2.2.1.1.9. Assessment of impact of ITN

Number of vector mosquitoes found dead on the floor sheets in the morning... (a)

Number collected alive by hand catch ... (b)

Number found dead in exit traps ... (c)

Number found alive in exit traps ... (d)

Number found dead after 24 h holding among the alive mosquitoes ... (e)

Total entry = a+b+c+d per room per night ... (f)

Comparisons between various treatments and control should be made

and the inferences should be drawn as under:

Immediate mortality = $[a+c/f] \times 100$

Indoor resting density = $a+b+c+d$

Excito-repellency = exit to entry rate $[(c+d)/(e+f)] \times 100$

Delayed mortality = $[\text{Number dead after 24 hrs among the alive mosquitoes} / (f)] \times 100$

Overall mortality = $[\text{Number dead on floor sheets, in traps \& 24 h holding} / (f)] \times 100$

2.2.1.1.10. Adverse effects, acceptability by community and collateral benefits

Information on these aspects will be generated by interviewing the inhabitants using structured questionnaires (Annexure 6).

2.2.1.1.11. Human safety

This should be accomplished by interviewing the inhabitants and applicators using structured questionnaire (Annexure 3). A registered medical practitioner should be associated for collection of data.

2.2.1.1.12. Criteria for selection of dosage of insecticide for Phase III trial

The minimum dosage of the insecticide that yielded the maximum reduction of human-vector contact as derived from the trial with village huts or experimental hut trial (Phase II) should be considered for Phase III evaluation.

2.2.1.2. Phase III trial

Duration: Three years

Objectives

- To assess the efficacy of the net treated with the selected application dosage against the target vector species
- To study the impact on disease prevalence
- To assess the persistent effect and treatment (impregnation) intervals of the net
- To assess community acceptability and collateral benefits
- To study safety to applicators and inhabitants

2.2.1.2.1. Selection of villages

For selection of villages, the section 2.1.3.1 may be referred.

2.2.1.2.2. Census of selected villages

Census should be conducted prior to intervention in the selected experimental and control villages and houses should be numbered and recorded in the format given in Table 23. This is to estimate the number of nets actually required for the trial.

Table 23. Record of census of households

Village..... Sub-centre..... PHC.....District.....State.....
House No.Type of the structure.....Date of survey.....

| S. No. | House No. | Name | Relation | Age/ sex | Education | Profes- sion | No. of rooms | Sleeping habits inside/outside | No. of nets required |
|--------|-----------|------|----------|-------------|-----------|-----------------|-----------------|--------------------------------------|-------------------------|
| 1. | | | | | | | | | |
| 2. | | | | | | | | | |
| 3. | | | | | | | | | |
| 4. | | | | | | | | | |

2.2.1.2.3. Treatment and distribution of nets

The required number of mosquito nets should be impregnated following the procedure described in Box 7. Informed consent should be obtained from the participants in the trial (Annexure 5). Inhabitants should be educated on the use and storage of nets and instructed not to wash the nets. All the nets should be marked with a water-soluble ink to detect whether the nets are washed or not. Every time the team goes for data collection the marking on the nets should be checked and recorded. To ensure the regular use of nets by the inhabitants, trained

volunteers from the same village should be engaged. Compliance of use of nets should be assessed fortnightly and recorded in the format given in Table 24. Sample size for coverage and compliance is given in Table 25.

Table 24. Record of compliance of treated nets in households

Village.....Sub-centre.....PHC..... District.....State.....

House No..... Type of the structure.....Date of survey.....

| House code (Arm No./ Insecticide dose) | Date and time of visit | Use of net | |
|--|------------------------|------------|----|
| | | Yes | No |
| 1. | | | |
| 2. | | | |
| 3. | | | |
| 4. | | | |
| 5. | | | |

Table 25. Sample size selection based on required confidence levels
Required Sample Size[†]

| Population Size | Confidence = 95% | | | | Confidence = 99% | | | |
|-----------------|------------------|------|------|------|------------------|------|------|-------|
| | Margin of Error | | | | Margin of Error | | | |
| | 5.0% | 3.5% | 2.5% | 1.0% | 5.0% | 3.5% | 2.5% | 1.0% |
| 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| 20 | 19 | 20 | 20 | 20 | 19 | 20 | 20 | 20 |
| 30 | 28 | 29 | 29 | 30 | 29 | 29 | 30 | 30 |
| 50 | 44 | 47 | 48 | 50 | 47 | 48 | 49 | 50 |
| 75 | 63 | 69 | 72 | 74 | 67 | 71 | 73 | 75 |
| 100 | 80 | 89 | 94 | 99 | 87 | 93 | 96 | 99 |
| 150 | 108 | 126 | 137 | 148 | 122 | 135 | 142 | 149 |
| 200 | 132 | 160 | 177 | 196 | 154 | 174 | 186 | 198 |
| 250 | 152 | 190 | 215 | 244 | 182 | 211 | 229 | 246 |
| 300 | 169 | 217 | 251 | 291 | 207 | 246 | 270 | 295 |
| 400 | 196 | 265 | 318 | 384 | 250 | 309 | 348 | 391 |
| 500 | 217 | 306 | 377 | 475 | 285 | 365 | 421 | 485 |
| 600 | 234 | 340 | 432 | 565 | 315 | 416 | 490 | 579 |
| 700 | 248 | 370 | 481 | 653 | 341 | 462 | 554 | 672 |
| 800 | 260 | 396 | 526 | 739 | 363 | 503 | 615 | 763 |
| 1,000 | 278 | 440 | 606 | 906 | 399 | 575 | 727 | 943 |
| 1,200 | 291 | 474 | 674 | 1067 | 427 | 636 | 827 | 1119 |
| 1,500 | 306 | 515 | 759 | 1297 | 460 | 712 | 959 | 1376 |
| 2,000 | 322 | 563 | 869 | 1655 | 498 | 808 | 1141 | 1785 |
| 2,500 | 333 | 597 | 952 | 1984 | 524 | 879 | 1288 | 2173 |
| 3,500 | 346 | 641 | 1068 | 2565 | 558 | 977 | 1510 | 2890 |
| 5,000 | 357 | 678 | 1176 | 3288 | 586 | 1066 | 1734 | 3842 |
| 7,500 | 365 | 710 | 1275 | 4211 | 610 | 1147 | 1960 | 5165 |
| 10,000 | 370 | 727 | 1332 | 4899 | 622 | 1193 | 2098 | 6239 |
| 25,000 | 378 | 760 | 1448 | 6939 | 646 | 1285 | 2399 | 9972 |
| 50,000 | 381 | 772 | 1491 | 8056 | 655 | 1318 | 2520 | 12455 |
| 75,000 | 382 | 776 | 1506 | 8514 | 658 | 1330 | 2563 | 13583 |
| 100,000 | 383 | 778 | 1513 | 8762 | 659 | 1336 | 2585 | 14227 |
| 250,000 | 384 | 782 | 1527 | 9248 | 662 | 1347 | 2626 | 15555 |
| 500,000 | 384 | 783 | 1532 | 9423 | 663 | 1350 | 2640 | 16055 |
| 1,000,000 | 384 | 783 | 1534 | 9512 | 663 | 1352 | 2647 | 16317 |
| 2,500,000 | 384 | 784 | 1536 | 9567 | 663 | 1353 | 2651 | 16478 |
| 10,000,000 | 384 | 784 | 1536 | 9594 | 663 | 1354 | 2653 | 16560 |
| 100,000,000 | 384 | 784 | 1537 | 9603 | 663 | 1354 | 2654 | 16584 |
| 300,000,000 | 384 | 784 | 1537 | 9603 | 663 | 1354 | 2654 | 16586 |

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2.2.1.2.4. Insecticide efficacy evaluation

Evaluation for insecticide efficacy should be carried out essentially following the procedure already described in the Phase II (2.2.1.1.6.). Fortnightly bioassays should be carried out throughout the duration of the Phase III. Nets for bioassays should be collected randomly and a minimum of four replicates should be used including control.

2.2.1.2.5. Entomological evaluation

The following evaluation should be done in the experimental and control villages at fortnightly intervals in randomly selected dwellings in corners and centre of the village.

2.2.1.2.5a. Floor sheet collection

For details see section 2.1.2.2.7.1.

2.2.1.2.5b. Exit window trap collection

For details see section 2.1.2.2.7.3.

2.2.1.2.5c. Hand catch

For details refer section 2.1.2.2.7.2.

2.2.1.2.5d. Mosquito landing collection on man

(Mosquito-landing collections on man may be done if feasible and on obtaining necessary clearance from human ethics committee)

All night collections of mosquitoes landing on man are made 10 days prior to distribution of nets and after 3rd and 6th months of distribution. Collections are made in two houses each in all the selected villages. Engaging volunteers as human baits inside the net, mosquito landing collections are made from dusk to dawn both indoors and outdoors of the selected houses. Informed consent of the volunteers should be obtained before the experiment (Annexure 4). Hourly mosquito collections should be recorded separately in the format as given in Table 15. Results are expressed as number of vectors per bait per night. The observations also provide information on biting rhythm (from the hourly collections) and feeding habit of the vector species in the area.

2.2.1.2.5e. Infection and infectivity rates

For details refer to section 2.1.3.7.1.3.

2.2.1.2.5f. Entomological inoculation rate (EIR)

For details refer to section 2.1.3.7.1.4.

2.2.1.2.5g. Blood meal analysis

To determine the feeding preference of vector species, blood smears from abdomen of fed mosquitoes are made on Whatman No. 1 filter paper. Blood samples numbered species-wise and structure-wise should be kept in plastic bags in desiccators before testing. Blood meal identification can be carried out by counter current immuno-electrophoresis (Bray et al 1984) with modifications of Collins et al (1986)/ ELISA. Results should be recorded in the format given in Table 26.

Table 26. Record of blood meal source

Village..... Sub-centre..... PHC..... District

Date of collection House No. Date of survey

| S. No. | Date of collection | Species and code | Result of blood meal source* |
|--------|--------------------|------------------|------------------------------|
| 1. | | | |
| 2. | | | |
| 3. | | | |
| 4. | | | |
| 5. | | | |

*H = Human blood; B = Bovine blood.

2.2.1.2.6. Disease prevalence

Data on point prevalence of malaria should be generated. Sample blood surveys are carried out both in experimental and control villages. The frequency and period of the survey can be determined based on the transmission pattern of the disease and in consultation with state health department. It is recommended that the first survey should be conducted 30 days prior to the distribution of nets and subsequent surveys should be conducted every three to six months after distribution. Surveys may be carried out following systematic sampling method selecting every 4th or 5th house depending on the total number of households to be selected in each village, which will be proportionate to the population size (PPS) of the villages. Rapid diagnostic kits (RDKs)/ microscopic examination of stained blood smears will be used to screen the inhabitants of the selected houses for malaria infection. The test/ microscopic positive persons will be administered with anti-malaria drugs as per the NVBDCP guidelines. The health workers of the respective PHC will be involved in the treatment of malaria positive persons. Data should be recorded in the format given in Table 27a & 27b.

Table 27. Mass blood survey

a. Data collection sheet

Village..... Sub-centre..... PHC..... District.....

Date of collection

| S. No. | H. No. | HOF | Name of patient | Age | Sex | Blood smear No. | Fever history | Drugs given | Result of smear | | |
|--------|--------|-----|-----------------|-----|-----|-----------------|---------------|-------------|-----------------|-----------|-----|
| | | | | | | | | | <i>Pv</i> | <i>Pf</i> | Mix |
| 1. | | | | | | | | | | | |
| 2. | | | | | | | | | | | |
| 3. | | | | | | | | | | | |
| 4. | | | | | | | | | | | |
| 5. | | | | | | | | | | | |

b. Summary Sheet

Village..... Sub-centre PHC District State.....
 Date of collection Insecticide & Dose..... Date of spray/round

Part 1

| S.No/ 1. 2. | Name of Village | HOF | Population | | | | | Total |
|-------------------|--------------------|-----|------------|-----|-----|------|-----|-------|
| | | | <1 | 1-4 | 5-8 | 9-15 | >15 | |
| 1. | | | | | | | | |
| 2. | | | | | | | | |

Part 2

| Blood smears collected | | | | | Drugs consumed | | | | |
|------------------------|-----|-----|------|-----|----------------|------|-----|-------------|-------------|
| <1 | 1-4 | 5-8 | 9-15 | >15 | Total | 4 AQ | 8AQ | Paracetamol | Other drugs |
| | | | | | | | | | |

Part 3

| Positives detected | | | | | % blood smears | | Positives | | | Remarks |
|--------------------|-----|-----|------|-----|----------------|-----------|-----------|-----------|-------------|---------|
| <1 | 1-4 | 5-8 | 9-15 | >15 | Total | collected | <i>Pv</i> | <i>Pf</i> | Mixed Total | |
| | | | | | | | | | | |

2.2.1.2.7. Significance of indicators

- Hand catch: Relative density of mosquitoes and other non-target insects
- Floor sheet collections: Immediate mortality
- Exit window trap collections: Excito-repellency of the insecticide
- Mosquito human landing collection: Man vector contact
- Infection and infectivity rates: Intensity of transmission
- Entomological inoculation rate: Force of transmission/ transmission load in the community
- Disease prevalence: Impact on incidence of malaria infection

2.2.1.2.8. Adverse effects, acceptability of community and collateral benefits

Collateral benefits of the insecticide treated nets should be assessed by interviewing the inhabitants using structured questionnaires for nuisance mosquitoes, bedbugs and human lice (Annexure 6).

2.2.1.2.9. Human safety

This should be accomplished by interviewing the subjects using structured questionnaire (Annexure 3). A medical practitioner should be associated for collection of data.

2.2.1.2.10. Operational acceptability and ease of impregnation

- The impregnated material should get into solution/suspension immediately and remain stable for sufficient period for proper impregnation
- The impregnated material should not cause irritation on contact with skin. Acceptability by community as determined by odour, effect on skin, etc., assessed by administering a structured questionnaire. (Annexure 6)
- Effect on non-target household pests/ insects and collateral benefits (Annexure 6)
- Safety to applicators and inhabitants (Annexure 3)

2.2.2. Evaluation of new insecticides for impregnation of nets

These are the insecticides evaluated for the first time. The specifications of the insecticide compound/ formulation should be provided by the manufacturer/ sponsoring agency together with material safety data sheet (MSDS) for toxicological indices on safety for humans and non-target organisms including domesticated pet animals.

The evaluation should be conducted in three phases - I, II and III. Phase II and III evaluation should be similar as described earlier for WHOPES passed insecticides (For details refer to section 2.2.1.1 and 2.2.1.2). Evaluation methods for Phase I are given below.

2.2.2.1. Phase I

2.2.2.1.1. Duration: 6 months.

2.2.2.1.2. Objectives

- To assess the efficacy and residual action of the insecticide on given net material
- To determine the effective dosage(s) of the insecticide for net impregnation

2.2.2.1.4a. Chemical assay

This is required to confirm the target dosage of insecticide in the treated net. The samples for the assay need to be labelled, kept in separate aluminium foils and stored in a cool place, prior to despatch for analytical laboratory. Care should be taken for correct measurement of area of netting. The quantity of the insecticide per unit surface area (ex. mg/m²) needs to be calculated.

2.2.2.1.4b. Determination of dosage for impregnation

Dosage for impregnation is essentially determined by exposing the laboratory reared 3-day old sugar fed female mosquitoes to treated nets using WHO cones (cone bioassays, Section 2.2.1.1.6.). To minimize the effect of overcrowding, in each cone only 5 females are to be

introduced. Two square metre pieces of mosquito net of different fibres namely polyester, nylon, High-density polyethylene (HDPE), cotton and polypropylene are impregnated following the procedure described earlier (Box 7). These fabrics are impregnated with different concentrations - 5, 10, 15, 20, 25, 30, 40, 50 mg/m². These pieces of impregnated nets are dried for a day or more and stored in a refrigerator at 4- 8 °C till use. These impregnated fabrics are used to determine the susceptibility status of the vector species by cone bioassays. At least two exposures should be made for each dosage and a control. Results should be expressed as average of two exposures. Abbott's correction should be made if needed as described in Table 22. Data should be recorded in the format given in Table 28. Percent mortalities observed after 24 h holding in exposures are regressed using probit analysis and from the dose mortality regression line dosage for 99.9% mortality is determined for the given fabric. This dosage can be used for assessing the residual action.

Table 28. Determination of dose for impregnation

Date of Exposure..... Test species..... Lab/ F₁/Field collected.....

| Dose (mg/m ²) | No. exposed | Fabric | | Average % mortality | Corrected % mortality |
|------------------------------|-------------|--------|----|------------------------|-----------------------|
| | | R1 | R2 | | |
| 5 | | | | | |
| 10 | | | | | |
| 15 | | | | | |
| 20 | | | | | |
| 25 | | | | | |
| 30 | | | | | |
| 40 | | | | | |
| 50 | | | | | |
| Control 1 | | | | | |
| Control 2 | | | | | |

2.2.2.1.5. Residual action on mosquitoes

Residual activity is determined following the methods described earlier (section 2.2.1.1.6.). Two square metre of the given net/ fabric is impregnated with the dosage already determined (LC₉₉). Cone bioassays using the target vector species are conducted one day after impregnation and subsequently at fortnightly/ monthly intervals. The bioassays should be carried out during the entire period of study. Bioassays are continued further for a few more months, if needed to assess the efficacy till 80% mortality is recorded and a confirmatory exposure is made to ascertain the results. The duration up to which the mortality is ≥80 % is considered as the effective duration of the given insecticide on the given fabric.

Phase II and III evaluations are to be performed as described for WHOPES passed insecticides (see sections 2.2.1.1 & 2.2.1.2).

2.2.3. Long-lasting insecticide treated nets

In these nets, insecticide treatment is done at the industry itself during the process of manufacturing. The net fibres are treated with insecticide following two techniques, 1. The insecticide is incorporated directly into the fibres and the insecticide diffuses to the surface with temperature and 2. The insecticide impregnated to the net is protected by a chemical (resin) coating thereby withstanding repeated washes, and thus these nets are called long-lasting insecticide treated nets (LLIN). The bioavailability of the insecticide on the surface of the net will be sufficient to be lethal to vector mosquitoes for extended periods (4- 5 years). The sponsoring agency should provide LLIN and its specifications.

Duration

Evaluation should be carried out for 20 WHO standard washes or for 3 years.

The evaluation of LLINs include

- Determination of bio-efficacy of the net in relation to number of washes
- Assessing the impact of LLIN on disease prevalence
- Assessing the collateral benefits of LLIN usage
- Studying the social acceptability through coverage and utilization and
- Assessing the human safety in accordance with increased coverage

2.2.3.1. Laboratory studies (Phase I)

Objective:

- To determine the efficacy of LLINs in relation to 20 standard washing

2.2.3.1.1. Impact of washing on bio-efficacy of LLIN (Wash resistance)

Each net will be washed according to the standard WHO washing protocol in a non-plastic bowl (metal or aluminum) containing 10 litre of water (water from a well or de-chlorinated water with a maximum hardness of 5 dh) and containing 2g/l of soap (locally available soap with ISI brand). The nets will be subjected to a manual agitation for 3 minutes, left to soak for 4 minutes and re-agitated for 3 minutes (total 10 min). Agitation will be done by stirring the net with a pole at 20 rotations per minute. Nets will be thoroughly rinsed twice in fresh water (10 l per rinsing) and dried horizontally in the shade. Nets will be stored at ambient temperature in polythene bags of suitable size) during the length of the regeneration time in days (time period required for regeneration of an LLIN with its initial biological activity after standard washing and holding at 30 °C) on a surface on ground (plastic sheet, cot etc) before the next wash. Regeneration time for the insecticide used in the LLIN will be provided by the industry/ sponsor.

The nets will be subjected to washing similarly for 20 times. Comparison of mosquito mortality should be made between washed LLIN, unwashed LLIN, washed plain net to evaluate the impact of washing on the efficacy of net.

The bio-efficacy is assessed after each wash (after the re-generation period, i.e. just before the next wash) using cone bioassay.

2.2.3.1.2. Cone bioassays

The cone bioassays are to be performed on the nets (minimum four nets) washed at intervals required for regeneration as per the procedure described in section 2.2.1.1.6. Bioassay is done after each wash up to 20 washes or more as necessary or until the mortality drops below 80%. The number of washes up to which the mortality and/or knock down (KD) was above the cut-off point (>80% mortality after 24 hours and/or above 95% KD after 60 minutes post-exposure) will be recorded. Same nets should be used for repeated washes and bioassays. The data are recorded in the format given in Table 29. In case, the tested LLIN falls below the cut-off point, the evaluation will continue until 20 washes are completed; a tunnel test will then be done.

Considering only the standard cone bioassay may sometimes underestimate the efficacy of LLIN, especially in the case of nets treated with insecticides that have a high excito-repellent effect, such as permethrin and etofenprox. Hence, the efficacy of LLINs washed 20 times in terms of mortality and blood feeding inhibition and that do not meet the criteria of the cone bioassay, will be tested in glass tunnels as described in section 2.2.3.4.2.6. 'Tunnel test',

The LLINs subjected to a minimum of 20 washes and caused a mortality of $\geq 80\%$ and a blood feeding inhibition of $\geq 90\%$ in tunnel tests will be considered for Phase II evaluation.

Table 29. Impact of washing on the bio-efficacy

Brand..... Date of evaluation..... Date of previous washing.....
 Name of interviewer.....

| Date* | No. of wash | Mortality after 24 h (cone bioassay) | | |
|-------------|-------------|--------------------------------------|---------------|------------------|
| | | Washed LLIN | Unwashed LLIN | Washed plain net |
| Replicate 1 | | | | |
| Replicate 2 | | | | |
| Replicate 3 | | | | |
| Replicate 4 | | | | |

2.2.3.2. Phase II evaluation (Experiment hut trial)

2.2.3.2.1. Objectives

The overall purpose is to determine the efficacy of the candidate LLIN washed 20 times against free-flying, susceptible wild mosquitoes in comparison to a negative control (an

untreated net with same netting material, mesh size and denier) and a positive control (a WHOPEs-approved LLIN with similar specifications such as insecticide, technique of impregnation, netting material, denier and mesh size) in experimental huts that simulate local domestic habitations.

The primary outcomes measured in experimental huts are:

- The deterrence (reduction in hut entry relative to the control huts provided with untreated nets);
- Induced exophily (the proportion of mosquitoes that will be found in the veranda);
- Blood feeding inhibition (the reduction in blood feeding compared with that in the control huts); and
- Immediate and delayed mortality (the proportion of mosquitoes that are found killed of the total numbers that entered early morning and after 24 hrs holding alive mosquitoes).

The first three of these outcomes will be indicators of personal protection, and benefit individual users. The fact that blood-seeking females are killed will be also important because community-wide use of treated nets can, in some circumstances, produce a “mass population effect”, i.e. a reduction in the density of infective mosquitoes in the area and, consequently, protection of the whole community, including those not using treated nets.

The personal protection effect of a treated net can be estimated by the calculation:

$$\% \text{ personal protection} = 100(B_u - B)/B_t$$

Where, B_u = is the total number blood-fed in the huts with untreated nets, and B_t is the total number blood-fed in the huts with LLIN /treated nets.

The potential mass effect of a treatment can be estimated by the calculation:

$$\text{Mass killing effect (\%)} = 100(K_t - K_u)/T_u$$

Where, K_t is the number killed in the huts with LLIN treated nets, K_u is the number dying in the huts with untreated nets, and T_u is the total collected from the huts with untreated nets.

2.2.3.2.2. Methodology

The study will be carried out using six experimental huts constructed in a village with abundance of the target vector species.

2.2.3.2.2.1. Design of experimental huts

The experimental huts are specially designed for recording the entering and exiting behaviour of mosquitoes and for measuring response to insecticides/treated nets including mortality. At

the end of the study, the huts can be renovated and used again. For the design of the experimental hut the section 2.1.2.1.1. may be referred. The number of huts to be constructed will be decided based on the number of arms (minimum four) included in the study and the number of replicates (four to six replicates) for each arm.

For acclimatization and to attract mosquitoes into the experimental hut, an adult volunteer will be enrolled for this purpose and he will sleep under an untreated mosquito net in huts from dusk to dawn for a period of 15 days. Clearance from ethical committee should be obtained to involve human volunteers in the study.

2.2.3.2.2.2. Pre-hut trial assessment

Subsequently, the suitability of the experimental huts will be assessed based on the following criteria over a period of one month prior to starting the hut-evaluation.

- i. Comparable indoor resting of mosquitoes with native village huts:
- ii. Tightness of huts (from recovery rate):
- iii. Absence of scavengers:

Details of assessment of the three criteria are given in the section: 2.1.2.1.2.

2.2.3.2.2.3. Maintenance of the huts

Rotation of the arms (test nets) will be done every four to six days. On the 5th or 7th day the huts will be cleaned and ventilated to remove contamination from the nets previously used. The huts are surrounded by a water-filled moat to exclude ants and other scavengers.

2.2.3.2.2.4. Organization of trial

Time Schedule

Hut evaluation will be done over a period of 6 weeks (6 weeks x 6 days) or 12 weeks depending on the availability of the vector mosquitoes in adequate number following a preparatory period as detailed below:

| | |
|---|-----------|
| Washing of nets: | 05 months |
| Transportation to field site, acclimatization of experimental huts and assessment of their suitability: | 02 months |

Experimental Arms

Unwashed and 20 times washed candidate **LLINs** will be evaluated in experimental huts for their effects against free-flying, wild mosquitoes and for their ability to deter entry, repel or drive mosquitoes out of houses, inhibit blood-feeding and induce mortality.

Untreated nets of the same material will be used as a negative control. WHO approved any other LLIN (with the same or similar specifications) **washed 0** and **20 times** shall be used as the positive controls. A **conventional ITN** (treated with the same insecticide at WHO recommended dosage) washed to just before exhaustion shall act as a reference net. The point of exhaustion should be determined at the field site by washing the conventionally treated net using the Phase II protocol. WHO cone bioassays will be performed after each wash. The last wash for which the net still causes $\geq 80\%$ mortality or $\geq 95\%$ KD will be considered to be the number of washes required before exhaustion. Normally, the nets will be of the following size: 205-220cm long, 170-175cm wide, 150-155 cm high. The nets and the insecticide will be provided by the industry/ sponsor.

In the WHO (2013) Guidelines for laboratory and field-testing of long-lasting insecticidal nets, the arm of conventional ITN (treated with the same insecticide at WHO recommended dosage) washed to just before exhaustion is not included for comparison with the candidate LLIN in Phase II evaluation. Therefore, instead of this arm, candidate LLINs washed more than 20 times, depending on the manufacture's claims, can be included as additional arms in the trial.

Preparation of nets (This is based on an example of six armed trial):

The nets will be coded (Codes X1, X2, X3, X4, X5 and X6 to indicate the six arms and letter code A, B, C, D, E, and F to indicate the six replicates of each arm, and Y and Z to indicate the additional nets of the arms, Y before any wash, Z washed until just before exhaustion or washed 20 times) by a member of the research team who will not directly be involved in the evaluation of nets in experimental huts and the codes will not be communicated to the field supervisor and field workers.

Six replicate nets will be used per arm and each net will be tested one night per week; (e.g. X1A, X1B, X1C, X1D, X1E, X1F, in which X1 will be the experiment arm and A, B, C, D, E and F will be its replicates).

2.2.3.2.2.5. Washing:

Refer to the section 2.2.3.1.1.

2.2.3.2.2.6. Bioassays

Bioassays and chemical analysis will be performed on the same nets on adjacent pieces of nets. Using the WHO prescribed cones; bioassays will be done on the nets with non-blood fed, susceptible vector mosquitoes. Cone bioassay before any wash and after washing 20 times or washing until just before exhaustion will be conducted. Also, one net per arm will be bio-assayed using non-blood fed susceptible vector species just before the field experiment (one randomly selected net out of the six replicate nets, A, B, C, D, E or F of each arm). One net per arm will be bio-assayed at the end of the field experiment with nets used in the huts (one randomly selected net out of the six replicate nets).

5 x 2 cone tests will be performed per net (on each section of the net: roof and 4 sides) (as shown in the figure below). Five female mosquitoes will be exposed per cone test

Exposure to net will be for 3 minutes after which mosquitoes are held for 24 hours with access to cotton wool pads soaked in 10% glucose solution. KD is recorded 60 minutes after the exposure time and mortality after 24 hours. Results are pooled for the 50 mosquitoes tested per net.

For baseline tests, results of the 5 locations on nets will be analyzed. After washing of nets, data of position 1 on the net will be considered separately and may have to be excluded since net at this position may have been subjected to abrasion in routine use.

2.2.3.2.7. Chemical assays

Prior to any wash, 5 pieces of 30 X 30 cm nettings (i.e. one piece each from positions 1 to 5) will be taken from one of the two additional nets (X1-6Y) of each of the six arms (Figure 1). Similarly, net samples will be obtained after 20 washes or after washing until just before exhaustion. At the end of the experimental hut study, one used net will be sampled in the same ways as described above. The samples will be labelled and packed in aluminium foil and stored at 4°- 8° C until sending them for chemical analysis to any laboratories (Govt./Pvt.) identified by the Directorate of NVBDCP/ CIB & RC. For chemical assay of washed nets, the net pieces cut from position number 1 will be analyzed separately.

2.2.3.2.8. Procedures for tests in huts

Tests will be done in the six experimental huts; in each hut one net will be used. Holes will be made in all nets of the six arms (six nets per arm) that will be used in the experimental huts to simulate the conditions of a torn net and to put emphasis on testing whether the insecticidal treatment, rather than the net, effectively prevents biting on the sleepers. Six holes (4 cm x 4 cm) will be made in each net, two each in long sides and one each in front and hind ends (Figure 2).

Figure 1. Pieces of nettings cut for chemical assay and bioassays

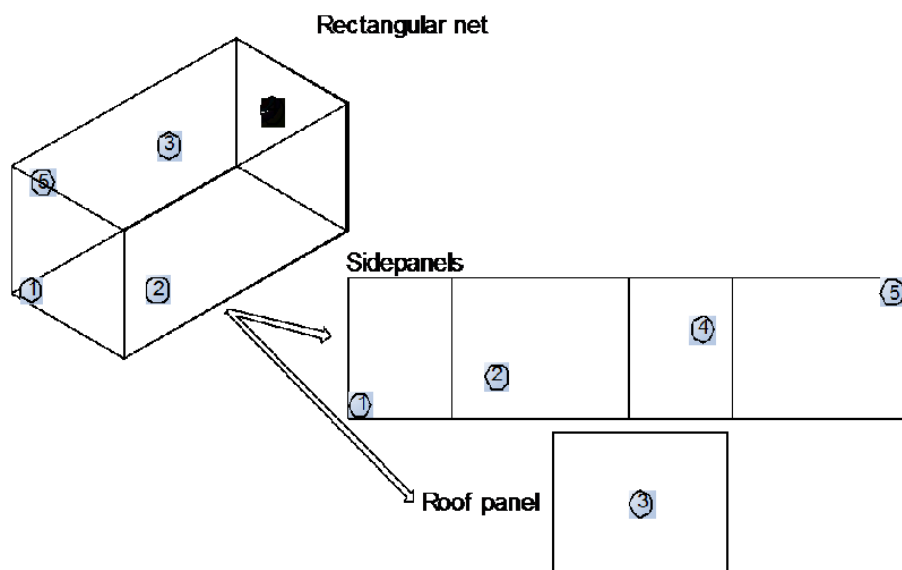
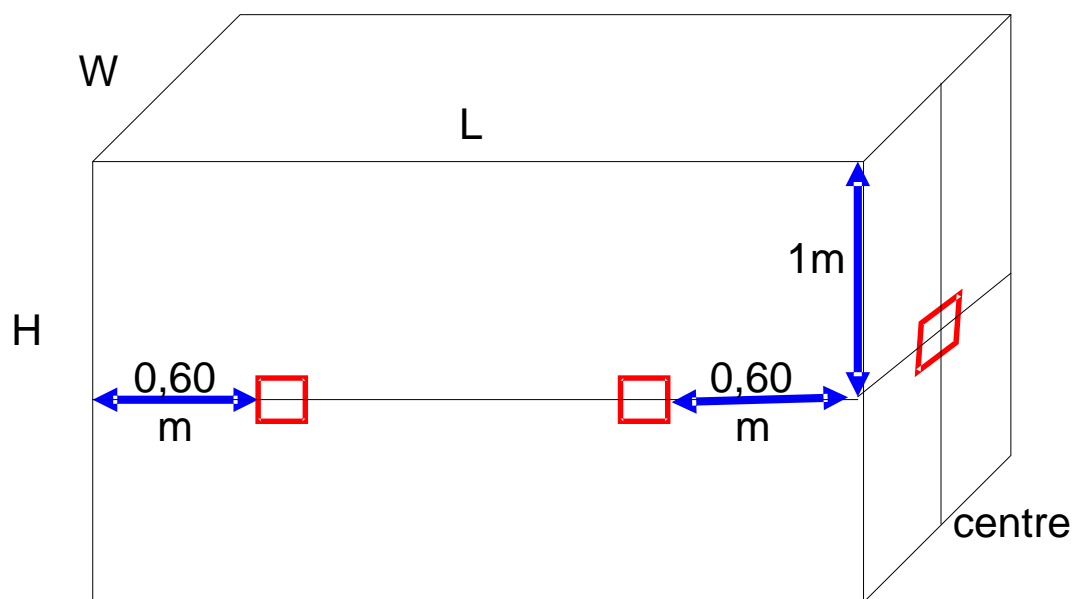


Figure 2. Holes made in nets tested in experimental huts



2.2.3.2.2.9. Procedure to be adopted by the volunteers sleeping under the nets inside the experimental huts

To sleep in the experimental huts, 12 volunteers, two (mostly husband and wife) per hut, will be selected in consultation with local village committee. The teams formed at the start of the study will not be changed unless any volunteer withdraws from the study.

The peripheral blood smears of the volunteers will be collected and examined in laboratory for the presence of malaria parasites. Those having malaria will be treated free of cost before they are included in the study.

The volunteers will be supplied with bedding set for sleeping.

The volunteers enter the experimental huts at 19.00 hours and remain inside until 05.30 hours. Inside the hut, they will sleep under the net assigned to that hut. Each volunteer will be compensated as per the human ethics guidelines.

In the evening before the volunteers enter the hut for sleeping, white cloth sheets will be spread on the floor of the hut and verandah after cleaning them and the moat around the hut will be filled with water.

Volunteers will be asked not to smoke or make fire inside the hut.

The treatment arms will be rotated among the huts each week according to the Latin square scheme, which will result in rotation of the volunteers each night to sleep under a different type of net. The volunteers will be asked to report any adverse events associated with use of any net as mentioned in the informed consent form for the net users (Annexure 3) and necessary medical care will be provided.

2.2.3.2.2.10. Rotation of treatments and volunteers

The nets and volunteers will rotate between huts using the Latin Square Rotation scheme (as shown below). The purpose of rotation is to minimize the variation caused by differences in attractiveness of huts (due to position) and sleepers. In practice, sleepers will rotate daily whereas experiment arms weekly.

In the morning, after collecting resting and dead mosquitoes, the nets will be removed and stored in their corresponding labelled cotton bag.

At the end of each week nets will be removed from the hut. The huts will then be cleaned and ventilated to remove any contamination from the nets previously used. The mat and the beds (labelled according to treatment) will be rotated with the respective arms since they come in close contact with the treated net. The treatment is then rotated to a different hut. The trial should continue for a multiple of 6 weeks to ensure complete rotation through the huts. In most cases, one or two complete Latin squares should be long enough to obtain sufficient number of mosquitoes for adequate statistical analysis.

Latin Square ROTATION scheme for experiment arms, nets* and sleepers

| Week | Day | Rotation of experiment arms | | | | | | Rotation of teams | | | | | |
|------|-----|--|--------|--------|--------|--------|--------|---------------------------------------|--------|--------|--------|--------|--------|
| | | Hut H1 | Hut H2 | Hut H3 | Hut H4 | Hut H5 | Hut H6 | Hut H1 | Hut H2 | Hut H3 | Hut H4 | Hut H5 | Hut H6 |
| 1 | 1 | X5 | X6 | X3 | X1 | X4 | X2 | F | C | B | E | D | A |
| | 2 | X5 | X6 | X3 | X1 | X4 | X2 | A | E | D | F | C | B |
| | 3 | X5 | X6 | X3 | X1 | X4 | X2 | B | F | A | D | E | C |
| | 4 | X5 | X6 | X3 | X1 | X4 | X2 | D | B | F | C | A | E |
| | 5 | X5 | X6 | X3 | X1 | X4 | X2 | E | A | C | B | F | D |
| | 6 | X5 | X6 | X3 | X1 | X4 | X2 | C | D | E | A | B | F |
| | 7 | Ventilating, cleaning and washing of hut | | | | | | No volunteers sleeping inside the hut | | | | | |
| 2 | 8 | X2 | X4 | X6 | X3 | X1 | X5 | A | B | E | C | D | F |
| | 9 | X2 | X4 | X6 | X3 | X1 | X5 | C | E | D | F | A | B |
| | 10 | X2 | X4 | X6 | X3 | X1 | X5 | F | C | A | D | B | E |
| | 11 | X2 | X4 | X6 | X3 | X1 | X5 | B | D | C | E | F | A |
| | 12 | X2 | X4 | X6 | X3 | X1 | X5 | D | A | F | B | E | C |
| | 13 | X2 | X4 | X6 | X3 | X1 | X5 | E | F | B | A | C | D |
| | 14 | Ventilating, cleaning and washing of hut | | | | | | No volunteers sleeping inside the hut | | | | | |
| 3 | 15 | X6 | X1 | X4 | X5 | X2 | X3 | A | D | F | E | B | C |
| | 16 | X6 | X1 | X4 | X5 | X2 | X3 | D | B | A | C | F | E |
| | 17 | X6 | X1 | X4 | X5 | X2 | X3 | B | F | E | A | C | D |
| | 18 | X6 | X1 | X4 | X5 | X2 | X3 | E | C | D | B | A | F |
| | 19 | X6 | X1 | X4 | X5 | X2 | X3 | C | E | B | F | D | A |
| | 20 | X6 | X1 | X4 | X5 | X2 | X3 | F | A | C | D | E | B |
| | 21 | Ventilating, cleaning and washing of hut | | | | | | No volunteers sleeping inside the hut | | | | | |
| 4 | 22 | X1 | X3 | X2 | X4 | X5 | X6 | E | C | B | F | A | D |
| | 23 | X1 | X3 | X2 | X4 | X5 | X6 | A | E | F | D | C | B |
| | 24 | X1 | X3 | X2 | X4 | X5 | X6 | F | B | E | C | D | A |
| | 25 | X1 | X3 | X2 | X4 | X5 | X6 | D | F | C | A | B | E |
| | 26 | X1 | X3 | X2 | X4 | X5 | X6 | C | D | A | B | E | F |
| | 27 | X1 | X3 | X2 | X4 | X5 | X6 | B | A | D | E | F | C |
| | 28 | Ventilating, cleaning and washing of hut | | | | | | No volunteers sleeping inside the hut | | | | | |
| 5 | 29 | X3 | X5 | X1 | X2 | X6 | X4 | D | C | B | F | E | A |
| | 30 | X3 | X5 | X1 | X2 | X6 | X4 | C | E | A | B | D | F |
| | 31 | X3 | X5 | X1 | X2 | X6 | X4 | B | A | F | D | C | E |
| | 32 | X3 | X5 | X1 | X2 | X6 | X4 | F | B | E | C | A | D |
| | 33 | X3 | X5 | X1 | X2 | X6 | X4 | E | D | C | A | F | B |
| | 34 | X3 | X5 | X1 | X2 | X6 | X4 | A | F | D | E | B | C |
| | 35 | Ventilating, cleaning and washing of hut | | | | | | No volunteers sleeping inside the hut | | | | | |
| 6 | 36 | X4 | X2 | X5 | X6 | X3 | X1 | B | E | A | C | F | D |

| | | | | | | | | | | | | |
|----|----|----|----|----|----|----|---|---|---|---|---|---|
| 37 | X4 | X2 | X5 | X6 | X3 | X1 | D | C | F | A | E | B |
| 38 | X4 | X2 | X5 | X6 | X3 | X1 | F | B | D | E | A | C |
| 39 | X4 | X2 | X5 | X6 | X3 | X1 | C | A | E | B | D | F |
| 40 | X4 | X2 | X5 | X6 | X3 | X1 | A | F | C | D | B | E |
| 41 | X4 | X2 | X5 | X6 | X3 | X1 | E | D | B | F | C | A |

NETS*:

Arm X1, nets A,B,C,D,E,F**
 Arm X2, nets A,B,C,D,E,F
 Arm X3, nets A,B,C,D,E,F
 Arm X4, nets A,B,C,D,E,F
 Arm X5, nets A,B,C,D,E,F
 Arm X6, nets A,B,C,D,E,F

SLEEPERS:

A: Team A – two volunteers
 B: Team B – two volunteers
 C: Team C – two volunteers
 D: Team D – two volunteers
 E: Team E – two volunteers
 F: Team F – two volunteers

2.2.3.2.2.11. Collection and processing of mosquitoes

Refer to sections 2.1.2.1.7.3.and 2.1.2.2.7..

2.2.3.2.2.12. Statistical analysis

For details of analysis, refer to section 2.1.2.1.7.5.

2.2.3.3. Phase II evaluation with village huts

Duration: 6 months

Wherever, construction of experimental huts is not feasible, the phase II evaluation may be carried out in the existing village huts with minor modifications with the consent of the respective household heads, essentially following the same procedure as given under experimental hut trial, except that the rotation of sleepers may not be possible as is done in the case of experimental huts as the residents may raise objections to allow outsiders to sleep in their house.

2.2.3.4. Phase III evaluation

Duration: Three years

Multi-centric, at least in three eco-epidemiological settings

2.2.3.4.1. Objectives

1. To evaluate the insecticidal activity and fabric integrity of the candidate LLIN over 36 months in comparison with preferably a WHOPEs approved LLIN (positive control) or

conventionally treated mosquito nets using the same insecticide and under the same field conditions.

2. To assess washing mode and washing habits of LLIN and ITN by the householders, and
3. To assess community acceptability of LLIN versus conventionally treated nets.
4. To assess the collateral benefits to the users

2.2.3.4.2. Methodology

This will be a prospective study with nets as the unit of observation as well as randomization. Two types of nets can be compared, i.e., LLIN and conventionally treated net of same fabric (in case of polypropylene, polyester fabric can be considered for conventional treatment) with the same insecticide (ITN) at WHO recommended dosage or the candidate LLIN with a WHOPES-approved LLIN with similar specifications in terms of insecticide, technique of impregnation, netting material, denier and mesh size.

Insecticidal efficacy, wash resistance, fabric integrity and washing practices of the householders, as well as their perception on adverse effects, if any, will be assessed during the study. The ITNs will be evaluated for at least one year or until they fail to meet the cut-off efficacy criteria¹. The efficacy of LLIN distributed in the villages will be monitored up to three years of continuous use under the field conditions.

2.2.3.4.2.1. Selection of villages

The LLINs and ITNs to be evaluated will be randomly distributed in 10-12 villages selected on the basis of population size, malaria incidence, accessibility, community use of ITNs/nets and multiple ethnicities in consultation with the State/District Health Authorities. In total, 440 households shall be included in the whole study. Of these, 300 households shall be given one coded LLIN each, and another 140 households shall be given one coded ITN each. This means, 300 LLINs and 140 ITNs shall be distributed in the beginning. All remaining persons in the households shall be given non-coded LLINs. The sample size calculation has been made based on the following criteria according to the study protocol:

The selection of multiple ethnicities for inclusion in the study shall be made in such a way that either the whole community fits in the study size with at least 60 households (30 with each type of net) included in each of the communities; or if the population size in a given community is large, a stratified sampling approach shall be used to select clusters of 60 adjacent households

2.2.3.4.2.2. Census and baseline household survey

A census will be carried out in all the selected villages. Enumeration of all houses will be done and detailed census with the name, age and sex of every family member will be recorded in registers. A baseline household survey will be carried out in all the selected villages using a

¹WHO cut off criteria: The conventionally treated nets are considered effective as long as at least 80% of them meet the cut-off criteria for the WHO cone bioassay test, i.e., if mortality in the WHO cone bioassays remains >80% and/or knockdown >95%.

structured questionnaire. Information will be collected on size of the family, educational status, occupation, average family income, type of house, number of sleeping places in a house, availability of nets/ITNs, their usage pattern, washing practices etc. Respondents will be heads of households or their spouses or other adult representative. The data recorded in community registers and questionnaires in the field will be transferred into a computer data file in an MS Access format.

2.2.3.4.2.3. Community education and informed consent procedure

As part of the community entry activities, the assistance of community leaders and local health workers in the selected villages will be sought; (1) to obtain permission to use the community as a study site, (2) to inform the community members about the purpose of the study, consequent sampling procedure and replacement of sampled net with new ones and (3) to seek community acceptance for use. In addition, community level meetings shall be organized to educate all the people in the selected villages on the adverse consequences of malaria, the benefits of using treated/long-lasting nets, correct handling and use of nets in line with WHO recommendation² and the need for reporting any adverse events, if any, and to seek their support in successful conduct of the study.

Written Informed consent will be obtained from all heads of households to be enrolled in the study at the time of census survey when all potential households will be visited by a team of investigators before distribution of LLINs/ ITNs (refer section 2.2.3.4.2.2.). A draft consent form is attached vide (Annex 5). To obtain informed consent of illiterate people, the informed consent form shall be read and explained by a member of the investigating team in local language in the presence of a community witness. Upon their consent, such people will be asked to mark a thumb impression on the form and the witness will be asked to sign. The participants shall be informed of possible benefits of sleeping under treated nets. They will also be made aware of possible adverse events during the initial few days of using such nets and which may include one or more of the following: itching of skin, facial burning/tingling, paraesthesia (numbness or a loss of physical sensation and/or tingling of skin), sneezing, liquid discharge from nose, feeling of headache, nausea, eye irritation and tears, experience of bad smell, body rashes etc. They will be told that based on previous experiences such events are usually transient in nature, however, if needed, they will be advised to contact a member of the research team or a physician at a local health facility just in case there is a need for medical attention. They will be advised to report on all such events to a team member for record. The team member will also facilitate the treatment, if required.

Any potential participant who refuses to participate in the study will be advised to seek medical care at the nearest health facility upon observing any signs or symptoms of malaria. Alternative participants will be recruited in the study. Further, the initial sample size of the study will be adequate enough to take care of the mid-course withdrawals.

2.2.3.4.2.4. Withdrawal of participants

²WHO (2002). Instructions for treatment and use of insecticide-treated mosquito nets. WHO Document WHO/CDS/WHOPES/GCDPP/2002.4 available on WHO Internet at http://whqlibdoc.who.int/hq/2002/WHO_CDS_RBM_2002.41.pdf

If at any point of time during the study a participant decides not to participate any further, he/she will be allowed to do so. All such participants withdrawing from the study will be allowed to retain their net. Record of all such participants will be kept confidential.

The requirement of nets will be estimated considering the number of nets needed for baseline assays, replacements at the time of withdrawal of these nets for bioassays/ chemical assays, and as an exit strategy for the ITN group (i.e., to provide LLIN to all ITN households whose nets have worn out or lost). In case, the comparison net is another LLIN replacements may not be required.

Volunteer adult males will be recruited locally for the study for treatment of mosquito nets. They will be given training on appropriate method of impregnation of nets and observance of safety precautions. Their informed consent shall be obtained using an informed consent form developed following the WHO guidelines (Annexure 1). They will also be told that proper use of safety equipment will prevent/ minimize adverse events during impregnation of nets as mentioned in section 2.2.3.4.2.3. and that the project will ensure medical care should such a need arises. They will be provided with personal protective equipment (PPE). and bath soap for washing and bathing at the end of day's work. A first-aid box will be provided to the supervisor.

For conventional treatment, nets shall not be pre-washed. The nets will be treated individually in basins using the required quantity of the insecticide at the recommended dosage. The net to be treated will be unfolded and put into the treatment basin with the insecticide solution that has been prepared. The dipped net will be turned thoroughly in the solution for at least 2 minutes, removed and allowed to drip over the basin but not wrung. The treated nets will be dried lying flat on the ground.

2.2.3.4.2.5. Distribution of LLINs/ITNs

A random list of ID numbers required for the study according to the sample size will be produced by the statistician in SPSS to allocate to LLIN or conventional ITN. After random allocation of ID number to the two groups of nets (LLIN and ITN), the ID numbers would be written with wash-resistant ink on a piece of polyester band (dry clean label) fixed on each net. In addition, these bands on each net will also be marked with a water-soluble ink as a quality control for the assessment of washing. After the ID numbers had been fixed on nets, all nets will be re-sorted in ascending order of ID number making it impossible to identify the type of nets by the field workers or study participants. Only the Principal Investigator will have the net master list to ensure that neither the distribution team nor the participant villagers know whether the net is an LLIN or ITN. The net master list will inter alia include the following information: ID number, type of net, household number, and dates of net distribution, sampling and replacement, if any. LLIN or a conventional ITN will then be distributed to households in each of the selected villages. This information that the nets have been marked with water-soluble ink as well as the purpose of such marking will also be provided to the participants in the interest of transparency. People will be asked not to remove the ID labels from the nets. The number of nets for each household will depend on the household size and sleeping places

in order to obtain full community coverage with an LLIN/ITN and obviate the need of other vector control interventions in the village. The nets will be given free of charge to all households and receipts obtained from them.

At the time of distribution of ITN/LLIN, every headperson of the household will be informed about the need for reporting adverse effects, if any, after using the nets, as well as their appropriate use and maintenance. This procedure will be repeated every time a net is withdrawn for laboratory assays and replaced with a new one, as well as at the end of the study during the dissemination meetings.

2.2.3.4.2.6. Sampling of LLINs/ITNs

A. Chemical assays

Samples of the LLIN, as well as samples of the conventionally treated nets, will be subjected to chemical residue analysis in a recognized laboratory. To ensure that the target dose of the insecticide has been achieved, netting pieces will be cut at the beginning of the trial for baseline assays. Thereafter, netting pieces will be cut when ITNs get exhausted or at the end of one year, whichever is earlier, while pieces of LLIN will be cut at the end of year 3 or when they fail to meet WHOPES criteria.

Thirty nets from each research arm will be destructively sampled randomly at base line (at week 1) for chemical residue analysis and at the end of the study. This will require selecting 30 each of ID numbers from LLIN and ITN groups plus 2-4 extra numbers as possible replacements in case the selected participant could not be reached or the net had been lost to follow-up since the last visit. For the chemical assay, sampling should be performed according to the scheme given in Figure 1 & 2.

Thus, from each of the 30 sampled nets, four rectangular pieces of 30 cm x 30 cm size will be cut from positions 2, 3, 4 and 5 using sharp scissors.³ The sub-samples will be rolled up and placed in new, clean and labelled aluminium foil for storage at +4 °C temperature prior to dispatch to the laboratory for the chemical assay. In the testing laboratory, the four sub-samples of each net will be assembled as one sample for chemical analysis. The results will provide the average target AI content of the insecticide in the LLINs or ITNs under evaluation.

B. Biological assays

Insecticide susceptibility tests of wild mosquito samples

Blood fed and gravid females of the target vector species will be collected from houses in the study sites using aspirators and maintained in the laboratory to lay eggs that will be used for

³For chemical assays, the sampling will be done from positions 2, 3, 4 and 5 only. Sample from position 1 will not be taken since netting fabric at this position is subjected to excessive abrasion in routine use (this portion of net is frequently manipulated while tucking the nets under the bed/mattress).

rearing F₁ progeny for susceptibility tests. The laboratory reared 2 to 5 days old; non-blood fed F₁ females will be used for the insecticide susceptibility test at diagnostic concentrations to determine susceptibility level using the standard WHO kits (tubes) (WHO 1998) for the candidate insecticide.

Insecticide efficacy evaluation

The standard WHO procedure (cone bioassay) will be used for evaluation of insecticidal effect of ITN/LLINs (WHO, 2005a). Accordingly, at the start of the studies and every 6 months thereafter, 30 nets of each type (LLINs/ITNs) will be randomly drawn from the net master list by the principal investigator and used by the research team for collection of samples for cone bioassays.

To obtain a good representation from each net, five samples (25 cm x 25 cm) will be cut from each of the 30 randomly selected LLINs/ ITNs from positions 1 to 5 as shown in Figure 1 and used for the bioassays. Pieces will be cut as rectangles using sharp scissors. Bioassays will be done using cones on all the five pieces. On each netting sample, standard WHO cone will be placed and held in place using a plastic manifold. Five laboratory-bred 2 to 5 days old; non-blood fed F₁ progeny of adults (fully susceptible to the candidate insecticide) collected from the study sites will be introduced into each cone and exposed for 3 minutes. The test will be done twice on each of the five netting samples cut from a net the same or the next day. Thus, in all 50 mosquitoes will be exposed on each net. Thus, altogether 1500 mosquitoes (30 nets x 5 positions per net x 5 mosquitoes per piece x 2 replicates) will be exposed.

After the exposure, the mosquitoes will be removed gently from the cones and kept separately in plastic cups provided with cotton-wool moistened with 10% glucose solution. Knockdown will be recorded after 60 minutes and mortality after 24 hours. Mosquitoes exposed to untreated nets will be used as controls. The bioassays will be done at 27±2 °C and 80±10% RH. Data will be recorded in a structured form for further analysis.

The bioassays will be done once at the start of the study as explained above, and every 6 months thereafter up to 3 years. Bioassays on ITNs will be done up to 1 year or until the biological activity declines below the recommended level (this will require a number of bioassays within the first year to determine when nets give knockdown rate of <95% and or mortality <80%). Nets selected randomly and withdrawn from a household for destructive sampling will be replaced with a new LLIN and the household will be excluded from the study for future sampling. Data for position 1 will be analyzed separately from those of other positions (no. 2 to 5) considering that netting at position 1 is subject to excessive abrasion in routine use. If there are significant variations in bioassay results, mean results of positions 2 to 5 will be used.

Tunnel test

LLINs or ITNs which cause a knockdown rate of <95% and a bioassay mortality of <80% will be subjected to a tunnel test. The tunnel test will be carried out in the laboratory, by releasing non-blood fed female anopheline mosquitoes, aged 5–8 days, in a tunnel (square section 25 cm

x 25 cm) made of glass, 60 cm length (WHO, 2005b). At each end of the tunnel, a 25-cm square mosquito cage covered with polyester netting is fitted. At one third of the length, a disposable cardboard frame is placed with the LLIN netting sample. The surface of netting “available” to mosquitoes is 400 cm² (20 cm x 20 cm), with nine holes each 1 cm in diameter: one hole is located at the centre of the square; the other eight are equidistant and located at 5 cm from the border. In the shorter section of the tunnel, an animal bait, small rabbit is placed, which will be unable to move but available for feeding. In the cage at the end of the longer section of the tunnel, 100 female mosquitoes will be introduced at 18:00. The following morning at 09:00, the mosquitoes will be removed by using a suction glass tube and counted separately from each section of the tunnel and mortality and blood feeding rates will be recorded (Annexure 7).

During the test, tunnel will be placed in a place maintained at 27 °C ± 2 °C and 80% ± 10% relative humidity. Several tunnels will be used simultaneously, one tunnel with untreated netting always being used as a negative control. Blood feeding inhibition will be assessed by comparing the proportion of blood-fed females (alive or dead) in treated and untreated tunnels. Overall mortality will be measured by pooling the mortalities of mosquitoes from the two sections of the tunnel.

Results of the cone and tunnel tests will be considered together to judge on LLIN performance. A candidate net will be deemed to meet the requirements of a LLIN if at the end of the study period of 3 years, at least 80% of sampled nets will retain bio-efficacy based on WHO cone bioassay and/or the tunnel test as detailed in the WHO guidelines.⁴

As blood-feeding in controls has a considerable impact on mortality in the presence of treated samples (i.e. the host-seeking behaviour increases the chance of contact with treated fabric), a minimum cut-off value of blood-feeding rate in controls will be established for tunnel tests.

2.2.3.4.2.7. Impact on disease prevalence

Refer to section 2.2.1.2.6.

2.2.3.4.2.8. Physical inspection of nets

The physical integrity of the net (e.g. size and number of holes) to be sampled will be determined by draping the nets over a frame and counting the number of holes and estimating their sizes according to the location on the net (top, upper side, lower side). The data will be recorded as per the questionnaire given in Annexure 8.

2.2.3.4.2.9. Assessment of community acceptance and practices

⁴Bio-efficacy criteria:

Cone bioassay: criteria for acceptance- mortality in mosquitoes >80% and/or Knock down >95%.

Tunnel Test: mortality in mosquitoes \geq 80% and blood feeding inhibition \geq 95%.

A team of staff will be trained on administering the questionnaire survey. Following assessments will be made:

(a). An assessment of adverse effects, if any, of ITNs among the net impregnators will be made using a questionnaire (Annexure 9). All the LLIN and conventional net impregnators will be interviewed at the end of the day of impregnation of mosquito nets, again in the following day morning and one week after completing the impregnation work.

(b). An assessment of adverse effects, if any, among ITN/LLIN users will be made using a questionnaire given in Annexure 10 during the periodic surveys. The Principal Investigator will select 30 ID numbers from LLIN group and another 30 from the ITN group from their respective master lists using a random selection procedure. The list of selected nets will be re-sorted in ascending order and given to the field team who will visit the study area one week and one month after distributing the nets to record perception of the participant users and to record any adverse effects. In addition, any such events reported proactively by the participants to the research team shall also be recorded and analyzed.

(c). At the end of months 1, 6, 18 and 30, an adult householder in 30 each of selected households will be interviewed by door-to-door visit to assess net utilization pattern/frequency of use (including early morning observations), method and number of washes and type of detergent used and physical integrity of the net (size and number of holes) as per the questionnaire vide Annexure 8. Since interview assessment of washing frequency may not always be reliable, another net in the household, or a net in a neighboring household, will be marked with a water-soluble marker and the household revisited one month later to obtain a more accurate assessment of the washing frequency in the community.

(d). Annual surveys (at the end of months 12, 24 and 36) of all the included households with remaining code LLINs/ITNs will also be conducted by visiting them door-to-door to record physical presence/absence and fabric integrity of the nets, where applicable, to estimate the annual attrition rate, besides information on people's perceptions and practices as mentioned above including any adverse effects observed. Questionnaires given in Annexure 8 and 10 will be used for these surveys too.

2.2.3.4.2.10. Interpretation of results / termination of the study

Each year, a formal report will be prepared and reviewed to take a decision on whether or not to continue the study for the next year. The decision will be made based upon the performance of the product in the field. If mortality in the WHO cone bioassays fall below 80% and or knockdown falls below 95%, nets will be tested in the tunnel test⁵. If mortality in the tunnel test falls below 80% and blood feeding inhibition falls below 95%, the net will be considered to have failed to meet WHOPES criteria. If >20% of the nets sampled fail to meet WHOPES criteria, the study will be stopped.

⁵Bio-efficacy criteria: Cone bioassay: criteria for acceptance- mortality in mosquitoes $\geq 80\%$ and/or knockdown $\geq 95\%$.

Tunnel Test: mortality in mosquitoes $\geq 80\%$ and blood feeding inhibition $\geq 95\%$.

2.2.3.4.2.11. Ethical clearance and considerations

The study will involve the ethical issue of protecting people's rights, possible inconveniences caused to them and protecting infringement of privacy of women during the study and more specifically during census and sociological surveys. The survey teams will preferably include a sociologist and a woman health worker to ensure that no infringement on human right occurs during the survey.

The study will not involve experimental use of animals. If it is necessary to conduct tunnel test to assess inhibition of mosquito feeding through LLIN/ITNs, rabbits will be used and they will be given due care as per standard practices. Also, the necessary ethical clearance will be obtained from the Animal Ethics Committee of the respective institution.

Note: Any household who withdraws from the study would be allowed to keep their LLIN/ITN.

2.2.3.4.2.12. Data entry and analyses

Data entered into the computer will have only codes of households and not names such that they are not easily identifiable to the study subjects by the data entry clerks. Furthermore, questionnaire data will be analyzed by inferential statistics (e.g. chi-square) to compare variables obtained. All information related to the participants will be kept confidential. The identity of the individual participant will not be revealed in any reports or publications resulting from the study.

Using the data obtained through questionnaire, community acceptance of LLINs (use rate, perceived benefits in malaria control, any adverse effects, washing and upkeep practices) and attrition rate will be assessed.

Data on adverse effects reported by the impregnators and users of LLINs and ITNs shall be separately analyzed and reported.

Results of the insecticide susceptibility tests (bioassay tests using WHO tube test and cone tests) will be analyzed for dose/response relationships (probit analysis) by the Maximum Likelihood method (Finney 1971). The differences in mortalities will be compared between LLIN and conventional ITN using the χ^2 statistic.

Using data of the tunnel test, blood feeding inhibition will be assessed by comparing the proportion of blood fed females (alive or dead) in treated and control tunnels. Overall mortality will be measured by pooling the immediate and delayed (24-hour) mortalities of mosquitoes from the two sections of the tunnel and the data will be interpreted using the criteria mentioned in section 4.2.10.

2.2.4. Insecticide incorporated plastic sheets & fabrics

Recently many products are being developed for protection from mosquito bites in complex emergencies. The insecticide is incorporated into the fibres of the fabric/plastic sheeting and the insecticide constantly diffuses out. The bioavailability of the insecticide on the surface of the fabric/sheet will be sufficient to be lethal to vector mosquitoes for extended periods. These are generally used in temporary habitations (tents) such as refugee camps, rehabilitation colonies, temporary shelters, etc. as tents.

Duration

Total duration of the trial is 21 months.

Objectives

- To determine the bio-efficacy of plastic sheets/ fabrics
- To study the impact of sheets/ fabrics on protection from mosquito bites
- To assess the collateral benefits
- To assess the social acceptability
- To assess human and usage safety

The evaluation should be done in the laboratory and field simultaneously.

2.2.4.1. Laboratory evaluation

Duration: Six months

The bio-efficacy of the insecticide incorporated plastic sheets & fabrics against vectors is assessed using cone bioassays. Cone-bioassays should be performed on Day 1 and at fortnightly intervals following the procedure described in section 2.2.1.1.6. Untreated plastic sheet/ fabric of the same material is kept as control.

2.2.4.2. Field evaluation

2.2.4.2.1. Duration: 15 months (covering all seasons)

2.2.4.2.2. Objectives

- To assess the efficacy on the prevailing disease vectors
- To study the impact on disease prevalence
- To assess community acceptability and collateral benefits
- To study safety of the fabric/sheet (human safety and fire safety)

2.2.4.2.3. Selection of localities and preparation of tents

Three localities having good vector productivity such as temporary habitations, slums, rehabilitation colonies, tribal hamlets, cantonment bases in remote areas should be selected. A minimum of six tents (6 x 5 x 2 m) should be laid at a distance of 5 metres with the given fabric/plastic sheeting. Control tents should also be prepared in the same locality or a different locality near-by with plain sheeting/fabric of the same material. Exit traps should be fixed to the nets.

2.2.4.2.4. Entomological evaluation

Evaluation should be done in the experimental and control tents at fortnightly intervals in randomly selected tents. Following evaluations should be done.

2.2.4.2.4a. Floor sheet collection

For details see section 2.1.2.2.7.1.

2.2.4.2.4b. Exit trap collection

For details see section 2.1.2.2.7.3.

2.2.4.2.4c. Hand catch collection

For details see section 2.1.2.2.7.2.

2.2.4.2.5. Disease prevalence

Disease prevalence studies should be carried as mentioned in section 2.2.1.2.6.

2.2.4.2.6. Significance of indicators

Hand catch: Relative density of mosquitoes and other non-target insects

Floor sheet collection: Immediate mortality

Exit window trap collection: Excito-repellency

Disease prevalence: Impact on incidence of malaria infection

Interpretation of data should be made as described in previous sections

2.2.4.2.7. Adverse effects, acceptability by the community and collateral benefits

These will be assessed by interviewing the inhabitants using structured questionnaires for nuisance mosquitoes, bedbugs, human lice, etc. (Annexure 6).

2.2.4.2.8. Human safety

This should be accomplished by interviewing the subjects using structured questionnaire (Annexure 6 two sets of questionnaire, one during the time of distribution and another at the end of third year of LLIN distribution). A medical practitioner should be associated for collection of data.



Net treated with insecticide



Experimental hut



Net cone-bioassay



2.3. Space Spraying

Space spraying is the dissemination of small droplets of insecticide (<50 µm) that will remain airborne for a considerable time (usually not more than 30 minutes) to make contact with the target species (WHO, 2010) and kill them instantly, but lack any residual effects. By killing adult mosquitoes, not only bites are prevented, but breeding is also prevented, resulting in net reduction in the mosquito population. There are two types of space spraying, 1) thermal fogging ($D_{v0.5} = 50 \mu\text{m}$) and ULV spraying ($D_{v0.5} = <25 \mu\text{m}$). The aim of space spraying is to rapidly reduce populations of flying insect pests and vectors. Since this type of treatment is not intended to leave a residual deposit, it involves a very low dosage of insecticide, but more frequent applications are usually needed to control the emerging adult populations. Space spraying is one of the options for the control of vectors, especially of dengue and malaria and also it is commonly used in public health pest control programmes against nuisance mosquitoes and flies.

The common protocol, revised based on the WHO Guidelines (WHO, 2009), provides precise and standardized procedures and criteria for testing efficacy and evaluation of insecticides for indoor and outdoor space spray applications against vectors and pests of public health importance. The WHO guidelines for development of an informed consent form are provided in Annexure 1.

The NVBDCP recommends thermal fogging, during disease outbreaks/ epidemics, both indoors and outdoors to mitigate the population density of disease vectors. It has the potential to be effective against peri-domestic breeding vectors. The effectiveness of fogging depends on dosage, size of spray droplets [1–50 µm ($D_{v0.5}$)], and flight activity of the targeted vector.

2.3.1. Phase I trial (laboratory evaluation) (for new insecticide molecules)

Duration: 3 months

New insecticides submitted by manufacturers/ importers will be directly subjected to Phase II and Phase III evaluation. For indigenous products, Phase I evaluation will be carried out in the laboratory. The determination of intrinsic insecticidal activity and diagnostic concentration for monitoring resistance will be carried out as described under sections 2.1.1.1 and 2.1.1.2. However, the insecticidal activity of the active ingredient(s) used as space sprays will be determined following method of WHO Guidelines (WHO/HTM/NTD/WHOPES/2009.2).

2.3.1.1. Insecticidal activity

2.3.1.1.1. Objective

To determine LC_{50} and LC_{90} of the given insecticide or its formulation against the target vector/ pest

In this test, the target species is exposed to the test concentrations of the atomized insecticide in a 'Wind tunnel'

- Ideally, five concentrations yielding mortality range between 10% and 90% should be tested; 2–3 below 50% and 2–3 above 50%.
- For each test concentration, duplicate cages of mosquitoes, each containing 25 non-blood-fed, 2–5 day-old susceptible female mosquitoes are used. A total of 100 mosquitoes are required for each test concentration and for the control.

The mosquitoes are exposed to one of the test concentrations of the atomized insecticide in a wind tunnel.

2.3.1.1.2. Description of ‘Wind tunnel’

The apparatus consists of a cylindrical tube (15.2 cm in internal diameter) through which a column of air moves at 2.9 m/s. The mosquitoes are confined in a rimless cylindrical screen cage (mesh openings 1.22 x 1.60 mm and 0.28 mm diameter wire) made to the exact interior measurements of the wind tunnel. The cage is inserted into an opening 91.4 cm from the wind tunnel entrance; a flexible clear plastic sheet is used to close the opening. For details of equipment specifications, maintenance and procedure, Annex 3 of the WHO Guidelines: WHO/HTM/NTD/WHOPES/2009.2. may be referred.

- The insecticide (technical grade) dissolved in acetone solution (0.5 ml total volume) is atomized through a nozzle (that takes approximately 3 seconds) to produce droplets with a $Dv_{0.5}$ of $15 \pm 2 \mu\text{m}$ at the position of the cage.
- Mosquitoes are held in the wind tunnel for a further 5 seconds. After each test, the mosquitoes are immediately transferred in to clean holding cups provided with 10% sugar solution. The cups should be held in dark place for 24 hours at $27 \pm 2 \text{ }^\circ\text{C}$ temperature and $80\% \pm 10\%$ RH. Mortality is recorded after 24 hours.
- Prior to testing of insecticide, control tests should be done using acetone alone as the diluents. The lowest concentration should be tested first and then proceeded with increasing concentrations.
- Between each series of concentrations, the wind tunnel is cleaned with a 0.5 ml spray of acetone.
- The test should be replicated three times using separately reared batches of mosquitoes and the results are combined for statistical analysis.
- Log-dose probit regression is used to analyze the relationship between concentration and mortality, as discussed above, and the LC_{50} and LC_{90} are determined.
- If control mortality exceeds 20%, the test is discarded and if the control mortality is between 5% and 20%, the test mortality is corrected using Abbott’s formula.

This procedure can also be used to test the formulations specially developed for space spraying. But, appropriate droplet size should be ensured by using a different nozzle. Wind tunnel testing is not suitable for most high volume thermal fog formulations (WHO/ HTM/ NTD/ WHOPES/ 2009.2).

2.3.2. Phase II trial (small-scale evaluation)

Duration: 6 months

Objectives

1. To determine the efficacy of the given insecticide formulation for space spraying indoors and outdoors
 2. To determine the optimum field application dosage of the insecticide for space spraying indoors and outdoors
- The efficacy of insecticides for space spraying is evaluated at small-scale in the field based on the mortality of laboratory-reared non-blood-fed female mosquitoes of susceptible strains confined in cylindrical cages (for specifications of bioassay cages, refer to Annex 6 in the WHO Guidelines: WHO/HTM/NTD/WHOPES/2009.2).
 - For indoor space spraying, cages are placed at 4-5 locations in rooms of 30m² and, for outdoor spraying, at downwind distances of 25, 50, 75 and 100 m.
 - Time of spraying and meteorological conditions (such as temperature, humidity, wind speed and direction) during the insecticide spraying should be recorded throughout the trial.
 - Prior to spraying, the delivery characteristics such as discharge rate, vehicle speed, nozzle angle and pressure need to be standardized.
 - The best conditions for outdoor space spraying usually prevail at or near dusk, throughout the night, and up to an hour after sunrise when temperature rises with height above ground level, i.e. inversion, when stable conditions keep the small insecticide droplets from rising above the target zone (WHO, 2009).
 - Small scale evaluation of space spraying outdoors should not be done when wind speed exceeds 15 km/hour or falls below 3 km/hour, or during rain (WHO, 2009).
 - Calibrations of sprayers is an important step to ensure delivery of droplets with a D_{v0.5} of desirable size, usually 15 ± 2 µm. In case of rapid volatility or other physical characteristic of the test material, the droplet size with D_{v0.5} of 10-40µm may be used.

2.3.2.1. Space spraying outdoors

Small scale outdoor trial is carried out with two primary objectives:

- To determine the dosage of active ingredient per hectare that causes at least 90% mortality and
- To physically characterize the spraying, mainly by assessing the droplet size.

Efficacy of the candidate insecticide should be assessed in an open field by observing the mortality of susceptible laboratory-reared 2-5 day-old female mosquitoes confined in screen cylindrical cages (size: 30 cm in diameter, 20 cm in height, with nylon mesh having 1.2 X 1.2 mm to 1.6 X 1.6 mm mesh openings) suspended 1.5 m above ground level. The cages are placed at 25, 50, 75 and 100 m downwind of the spray vehicle perpendicular to line of application of insecticide.

2.3.2.1.1. Physical characterization (WHO, 2009)

- Silicon coated slides (transparent collectors) are placed on rotators kept adjacent to selected cages to measure the droplet density and droplet size of the spraying (see Annexes 7 and 8 in the WHO Guideline, WHO/HTM/NTD/WHOPES/2009.2).
- Prior to spraying, the rotator is switched on. Fifteen minutes post-spraying, the rotator should be switched off and the collectors removed immediately (at the same time the bioassay cages are also removed).
- The collectors are then placed in a protective holder and transferred to the laboratory for droplet size assessment as soon as possible (considering the volatile nature of the formulation).
- Simultaneously, collectors should also be placed with the control cages in an unsprayed control area to detect the presence of any natural environmental droplets such as oils, plant sap etc. and separate these particles, if any, from the insecticide spray droplets.
- For non-volatile, oil-based insecticide formulations, collectors coated with silicone or Teflon can be used.
- For other types of formulations (e.g. water-based), slides/collectors coated with magnesium oxide can be used to detect the droplets provided a tracer dye is added to the spray, e.g. fluorescent tracer.
- The addition of tracer will make the smallest droplets visible as a distinct crater under an ultraviolet light microscope. Magnesium oxide coated surfaces are not suitable for measuring non-dyed droplet sizes of $<10\ \mu\text{m}$.
- The craters in magnesium oxide/ silicone- or Teflon-coated slides are then examined under a microscope and the droplet size is measured. The $Dv_{0.5}$ and $Dv_{0.9}$ are calculated (see Annex 8 in the WHO Guideline, 2009). For each collector, a minimum of 200 droplets should be measured reading across the width of the collector as many times as required.

2.3.2.1.2. Dosage determination (WHO, 2009)

- In small-scale field trials, a range of dosages of the given insecticide are tested based on label instructions and laboratory evaluation.
- The dosages should be selected in such a way that the range of efficacy produced by the dosages will include $>95\%$ mortality and at least one between 80% and 95% . It would be appropriate if the sequence of dosages is randomized and the spray equipment is cleaned with acetone between every applications.
- For each dosage, 1-2 cages will be placed at four distances i.e. 25, 50, 75 and 100 m in a row and a minimum of three such rows should be maintained. Each dosage should be tested on a minimum of three occasions.
- Cages are required to be positioned in the field for the standard exposure time of 15 minutes.
- For each replicate, unsprayed control with at least two cages and a rotating collector, should be kept in parallel at least 50 m upwind.
- A standard/ recommended insecticide can desirably be used as a positive control.

2.3.2.1.3. Stipulations

- The trial should be conducted in open areas where vegetation should not be taller than short grass so that the spray cloud will traverse through the sample line without any obstruction.
- The spray machine will be allowed to travel in a line that will be perpendicular to the direction of wind (testing should be avoided when wind directions are in excess of 30° from the sample line, because such condition significantly increases the distance between the spray line and the collection stations).
- There will be an increase in distance by 3.5% when wind direction is at 15° off-perpendicular and by 15.4% at 30°.
- At about 100 m distance before reaching the test area the spray machine should be turned on and it should be turned off at a minimum of 100 m beyond the test area.
- To assess the effect of spraying, 25 susceptible, non-blood-fed, 2–5 day-old laboratory-reared female mosquitoes of the target species are introduced in to each cage kept at the four distances in three rows.
- After the exposure time of 15 minutes (i.e. 15 minutes after completion of the spraying), the mosquitoes from each cage are transferred into clean holding cups marked separately, and provided with sugar solution on cotton wool (It should be ensured that from all the cages mosquitoes should be removed and transferred to the holding cups precisely after the exposure time to prevent excess exposure of mosquitoes to the insecticide residue in the cages).
- Number of mosquitoes knocked down at one hour post-spraying (including the 15 minutes exposure time) should be recorded.
- The mosquitoes are maintained at 27 ± 2 °C temperature and 80 ± 10 % RH and the number dead at 24-hour post-spraying is recorded.
- If the test is repeated on the same day, a minimum of 30 minutes should be the interval between each test, exposing mosquitoes obtained from different rearing cohorts.
- During each test, fresh dilutions of the formulation should be prepared and used.
- If the control mortality is >20%, the entire test should be discarded and repeated. If the control mortality is between 5% and 20%, the test mortality should be corrected to the control mortality using Abbott's formula.
- For each dosage the average mortality is estimated and recorded for each distance.
- If the spraying is done with vehicle-mounted machine, a dosage that causes an average mortality of >90% at all four distances is desirable and if it is a portable spray machine, the same level of mortality should be obtained at 25 m.

The results are recorded in the format as given in Table 30. For each dosage, the average mortality and its standard deviation are calculated and comparison is made between the dosages using an appropriate statistical test (e.g. ANOVA). The lowest dosage that causes 90% mortality should be the optimum dosage for operational trial.

Table 30. Efficacy evaluation

Village.....Sub-centre.....PHC.....District.....Date of bioassay.....
 Temperature: Min Max..... Relative humidity: MinMax.....
 Test species.....Lab/F1/Field collected.....

| Replicate* Cage No. | No. of mosquitoes in cage | No. knocked- down after 1 h | No. dead after 24 h | % knocked down after 1h | % Mortality after 24h |
|-----------------------------------|------------------------------|--------------------------------|------------------------|----------------------------|--------------------------|
| Replicate 1 | | | | | |
| Replicate 2 | | | | | |
| Replicate 3 | | | | | |
| Replicate 4 | | | | | |
| Replicate 5 (indoors) | | | | | |
| Control 1 | | | | | |
| Control 2 | | | | | |
| *Separate row for each replicate. | | | | | |

2.3.2.2. Indoor applications

Objective:

To determine the dosage of active ingredient of the insecticide/ formulation per cubic metre (m³) that causes at least 90% mortality.

- Indoor space spraying is evaluated in an empty room with a minimum volume of 30 m³, based on mosquito mortality observed in the test cages.
- Construction of experimental rooms is preferred to meet the test conditions. These rooms can be ventilated and adequately decontaminated.
- The effect of the indoor application is studied by placing a total of 10 cages inside the room, one cage 25 cm from each corner at ceiling and floor levels and two at mid height near the centre. In to each cage, 25 two to five days old non-blood-fed susceptible female mosquitoes are released.
- The room should be closed before spraying. The insecticide is released through an opening made at mid height in the centre of the wall at one end of the room and the nozzle of the spraying machine is directed towards the centre of the room.
- Before spraying, the spray machine must be calibrated to ensure a Dv0.5 of 15 ± 2 µm.
- At least three dosages of the insecticide should be tested. For each dosage, a minimum of three replicates should be kept, using insects from different rearing cohorts. Fresh dilutions should be prepared and used for each test.
- The mosquitoes confined in cages are exposed for 60 minutes and after the exposure time the cages are carefully removed. From each cage, the mosquitoes are quickly shifted to clean cups that are labelled separately and provided with a 10% sugar solution on cotton

wool for the mosquitoes to feed. While shifting, observation is made on the number of mosquitoes knocked down in each cage. To record the mortality at 24-hours post-spraying, the mosquitoes are maintained at 27 ± 2 °C temperature and $80 \pm 10\%$ RH.

- It is a must that the room is adequately ventilated between successive testing with different dosages of the same compound to remove all traces of the previous spraying.
- Control should be maintained for each insecticide application. This is done prior to spraying by exposing mosquitoes in cages for 60 minutes at each of the 10 positions as mentioned above.
- Before spraying, if the test mosquitoes held in a cage in the room for one hour show >10% knockdown/ mortality, the rooms should be declared contaminated or unfit for testing.
- The average mortality (with standard deviation) is calculated for each dosage and comparison is made between different dosages using appropriate statistical test (such as ANOVA).
- The lowest dosage that gives at least 90% mortality should be considered as the optimum dosage for operational trials.
- If the control mortality is >20%, the test should be discarded and repeated. If the control mortality is between 5% and 20%, the test mortality should be corrected to the control mortality using Abbott's formula (Table 31).

Table 31. Observed mortality of mosquitoes at different doses

| Type* | No. in cages | No. dead in cages | Total | | Mortality | Corrected % mortality |
|-------------------------------|--------------|-------------------|-------|-------|-----------|-----------------------|
| | | | Dead | Alive | | |
| Dose 1 | | | | | | |
| Dose 2 | | | | | | |
| Control | | | | | | |
| * Separate row for each dose. | | | | | | |

2.3.2.3. Human safety

Data regarding perception of the staff involved in space spraying and the community should be recorded in the pre-structured questionnaire (Annexure 3).

2.3.3. Phase III trial (Operational trial)

Duration: one year

- The operational should be a multi-centric study carried out at three eco-epidemiological settings covering different seasons.
- Based on the results of small scale trials, the effectiveness of the space spray product/ formulation should be assessed in operational settings against field populations of the target species.

- The study area should be representative of the target vector species' habitat and the intended control areas.
- The timing of spray and meteorological conditions (Section 2.3.2) should be recorded and delivery characteristics of the insecticide should also be standardized (Section 2.3.2). In addition, configuration of buildings, dwellings, rooms and vegetation characteristics in the study area should be surveyed and recorded prior to spraying of insecticide. Any other relevant observations such as time of sunset, sunrise, cloud cover and peak flight activity of target species should also be documented.
- The initial dosage for conducting operational trials should be determined based on dosage(s) recommended on the manufacturer's label or the dosage(s) that caused at least 90% mortality in small-scale trials.
- In the natural habitats (operational conditions), as there may be many obstructions and harbourages, spraying at higher dosages may be required.
- While spraying outdoors are based on dosage per hectare, indoor applications are based on dosage per cubic metre.
- Before conducting the operational trial, the susceptibility status of the target vector species to the test insecticide should be verified following the WHO procedures (WHO, 2009).
- Spray equipment must be calibrated to deliver a spray droplet distribution with a Dv0.5 of $15 \pm 2 \mu\text{m}$. In case of rapid volatility or other characteristics of the test material, larger or smaller droplets ranging from 10 - 40 μm (Dv0.5) may be required.
- As indicated under small scale trials, space spraying should not be carried out when wind speed exceeds 15 km/hour or falls below 3 km/hour or during rainfall. In most situations, a wind speed of approximately 3.6 -15 km/hour (i.e., 1-4 meters per second) is needed to drift the droplets downwind from the line of travel. Wind speed can be measured using a hand held anemometer.
- Outdoor space spray applications should be carried out when the meteorological conditions are favourable. The most advantageous conditions prevail when there is an increase in temperature with height. Ideally an inversion is needed i.e., colder air closer to the ground. This occur at or near sundown, throughout the night, and up to an hour after sunrise (WHO, 2009) Local knowledge of the time(s) of peak flight activity of the target vector species is essential for planning space spray treatments to coincide with the peak flight activity of the target species.

2.3.3.1. Trial outdoors

2.3.3.1.1. Objectives

1. To identify and confirm the effective dosage (required to achieve an average of 90% control) under operational settings and
2. To assess the impact of the space spraying on adult density of the target vector species.

2.3.3.1.2. Evaluation methods

- The guidance provided here is a general one and needs local adaptations considering the characteristic features of trial areas and behavioural differences among mosquito/vector species.
- The design of the trial should consider flight range and behavioural characteristics of the target species {e.g. indoor resting (endophily) and/or outdoor resting (exophily)} as well as the method of spraying.
- In operational trials, an adequately larger area should be sprayed to prevent/ minimize immigration/ re-invasion of the target species from unsprayed areas and evaluation of entomological parameters should be restricted to the central zone.
- The density of the target species should be monitored shortly before and immediately after spraying using appropriate sampling method to avoid the confounding influences of immigration or recruitment from the larval habitats of the study sites.
- For operational outdoor trial, the best suited design is random allocation of comparable treated and untreated areas. The trial should be replicated at least three times in space or preferably in time.
- In order to monitor the relative change in the density, appropriate adult sampling methods/devices to the target vector species should be used. These methods/devices should be employed in multiple representative habitat sites within the test and control areas (e.g. indoors or outdoors, or both).
- Collection methods may include the use of light traps, oviposition traps, gravid traps vacuum devices (aspirators) for resting mosquitoes.
- The sites identified for sampling and the collection methods should be configured in a way that maximizes the suitability for statistical analysis of data.

2.3.3.1.3. Evaluation using sentinel cages

- The spatial diffusion of spray cloud to the target test area is verified by observing mortality of mosquitoes confined in sentinel cages.
- The sentinel cages are placed at targeted areas within the spray sites (up to the distance where the spray cloud is expected to reach).
- For bioassays, batches of 25 non blood-fed, laboratory reared, 2-5 days old susceptible female mosquitoes are released into each of the sentinel cages.
- Adequate number of sentinel sites should be selected in a variety of open as well as sheltered habitats and replicated observations on mosquito mortality are made for statistical analysis.
- The experimental matrix design of sentinel cages in a treatment area as shown in Figure 4.1 in WHO Guidelines 2009 (WHO/HTM/NTD/WHOPES/2009.2) may be used. This design will allow the assessment of effects of two or more passes (multiple swaths) of outdoor space spray application.
- One sampling site should be selected from an unsprayed area 50 m upwind direction of the treatment zone and equal number of sentinel cages are placed for comparison.
- After exposure for 15 minutes, the cages should be removed and the mosquitoes are transferred to clean holding cups and held in an unsprayed room maintained at 27+ 2 °C and 60–70% RH.

- Mosquitoes should be provided with 10% sugar solution soaked cotton wool placed on the top of the cage. Percent mortality should be scored after exposure and after 24 h holding period.
- Along with the sentinel cages rotating collectors should be fixed to assess the droplet density and size.

Data analysis:

Data obtained from the sentinel cages and droplet collectors will be used for assessment of dispersion/ penetration of the insecticide Average mortality of mosquitoes in sentinel cage in a given area and the variation in the mortality between the cages should be estimated. To assess the efficacy of outdoor space spray treatment, the percentage reduction of wild caught mosquito populations between pre and post-treatment period as well as between treated and untreated areas will be estimated and compared.

2.3.3.2. Trial indoors

Evaluation methods

- Space spraying indoors is assessed by selecting several households, each with multiple rooms. Kitchens should be avoided.
- In each room, at least three cages (one cage should be placed near the centre at 1.5 m height and the other two cages should be placed adjacent to typical mosquito resting sites within each room). Twenty five (mixed age) wild caught female mosquitoes are released in each cage and exposed to insecticide application for 60 minutes in both experiment and control houses. A room fogged with only solvent is treated as control. If wild caught females are not available in sufficient number for bioassays, the F1 progeny of wild caught mosquitoes can be used.
- Before insecticide spraying, the external doors and windows of the house should be closed.
- In houses having more than one room, the farthest room from the entrance is sprayed first and progressively the other rooms moving towards the entrance. It should be ensured that the insecticide spray is directed to all parts of each room and at the target dosage.
- At least three applications (replicates) should be carried out on different occasions at the optimal dosage (required for 90% control) as determined in small-scale trials or at the label recommended dosage. Fresh dilutions should be prepared and used (if the formulation requires dilutions) for each replication.
- If the insecticide spraying does not cause the desired cage mortality of 95%, a higher dosage needs to be similarly tested. In such situation, the manufacturer's labeling constraints should not be exceeded.
- After the exposure of mosquitoes for 60 minutes, the cages are removed from the house. The cages should be handled carefully wearing adequate protective clothing. The mosquitoes are quickly shifted from the cages to clean separately marked holding cups provided with a 10% sugar solution. At the time of shifting, number of mosquitoes knocked down is recorded.
- The house should be ventilated by opening doors and windows before allowing unprotected individuals into the house.

- The mosquitoes in the holding cups are maintained at 27 ± 2 °C and $80 \pm 10\%$ RH and mortality is recorded at 24-hour post-spraying.



Fogging with a vehicle mounted sprayer

2.4. Repellents

Repellents are either synthetic chemical or plant-based compounds. They are used for personal protection against mosquitoes and other haematophagous insects. Candidate compounds should be evaluated simultaneously in laboratory and field.

2.4.1. Laboratory evaluation

2.4.1.1. Objectives

- To establish dose-response lines and effective doses (EDs) of a repellent corresponding to ED₅₀ and ED_{99.9} protection from mosquito landing/probing.
- To test the repellent activity of the candidate compound against mosquitoes
- To determine the complete protection time rendered by the repellent
- To test the safety to volunteers

2.4.1.2. Repellency against mosquitoes

These studies will be carried out in the laboratory maintained at 27±2°C and 80% ± 10% relative humidity using laboratory-reared strains of vectors in cloth cages (2 cubic feet). Studies will be carried out against each individual species in replicates of three. In each of the replicate cage, one hundred 3 to 5-days old sugar-fed female mosquitoes will be held. Mosquitoes that are pre-starved for 12 h or more prior to testing will be used for the experiment. These conditions and format of experiment given below need standardisation for different species. Use of positive control is essential in these tests. DEET (N,N-diethyl-3-methylbenzamide), the active ingredient of most commercially available repellents can be used as a positive control against which the effectiveness of the candidate repellent is tested.

In the cage, five plastic bowls with sugar-soaked cotton (10% in water) should be placed at four opposing corners and one in the middle. These bowls should be treated separately with three different specified concentrations of the formulation, one specified concentration of DEET (known synthetic repellent compound as positive control) and with only sugar-soaked cotton (negative control). The four treated bowls will be placed in four opposing corners while the untreated negative control in the middle on the floor of the cage. Five minute landing counts will be made at 0, 1, 2, 4 and 6 h. The cups will be removed between the exposure intervals. Mean percent repellency for each percent formulation and species will be calculated based on the data of the three replicates at the given times of observation. Percent repellency will be calculated using the formula given below.

$$\begin{aligned} & \text{(a) No. landing on negative control – No. landing on treated with repellent} \\ & \text{-----} \times 100 \\ & \text{No. landing on negative control} \\ & \text{(b) No. landing on negative control – No. landing on treated with DEET} \\ & \text{-----} \times 100 \\ & \text{No. landing on negative control} \end{aligned}$$

Where, (a) provides % repellency of the candidate repellent and (b) % repellency of DEET. Efficacy of the candidate repellent can be assessed relative to DEET

Studies with human volunteers should be undertaken only after ascertaining human safety.

2.4.1.3. Determination of effective dosage and protection time

(a) Effective dosage

Evaluations should be carried out in the laboratory maintained at standard temperature and humidity. Ideally, testing of repellents should be carried out on human subjects as they are the ultimate end-user of these compounds. The results of such tests will reflect the actual conditions of use. Therefore, the compounds that are toxicologically safer to humans should be evaluated on human subjects. The adult human volunteers exhibiting mild or no sensitivity to mosquito bites may be selected. Inclusion of equal numbers of male and female test volunteers is recommended. As a preparatory measure for the laboratory studies, the test area of the volunteer's skin should be washed with unscented soap and rinsed with water, then rinsed with a solution of 70% ethanol and dried. The test volunteers should avoid the use of any fragrance and repellent products at least for 12 hours prior to and during testing. Preferably, the volunteers should not be tobacco and alcohol users. Otherwise they should have refrained from tobacco and alcohol use at least for 12 and 24 hours, respectively prior to testing.

Testing should be done for Anopheles, Aedes and Culex mosquitoes and other disease vectors. Standardised mosquito rearing and colonising facilities are required to ensure reliability and reproducibility of data. In the laboratory, the stock population of adult mosquitoes containing both sexes should be maintained in the same cage to facilitate mating process. The stock population of adult mosquitoes should be provided with 10% sugar solution soaked on cotton-wool. From the stock population cage, host seeking female mosquitoes of uniform age (preferably 5-7 days) old should be selected for the test purpose. Prior to testing of repellence, the female mosquitoes were allowed to starve at least for 12 hours. In each test cage, about 50-100 active host-seeking females should be released in a 2 x 2 x 2 ft cloth cage. Observation of repellence should be made during times in the diel period that correspond with biting activity of the target mosquito species.

Serial dilutions of the candidate repellent are prepared with ethanol or other suitable diluents and tested to identify an effective dosage range. Ideally, five concentrations providing repellent responses between 10% and 90% are selected for this test, 2-3 dosages below 50% and 2-3 dosages above 50%.

Using a volunteer, at least five successive applications of incremental dosage can be tested. All the five applications should be tested in a single day using the same batch of mosquitoes by the same volunteer. The test will simultaneously be conducted using a minimum of three volunteers. The tests are repeated for at least three times using different batches mosquitoes on different days. Exposures are made sequentially of the forearm with application of diluents alone followed by progressively increased dosages of repellent.

About (~) 600 cm² area of one forearm skin between wrist and elbow of human volunteer should be marked with marker. One ml of ethanol/the same diluents used in the preparation of the test repellent is pipette out and applied evenly to $\approx 600\text{m}^2$ of the forearm skin between the

elbow and wrist and allowed to dry for about one minute. Prior to the insertion of the arm into the test cage containing unfed female mosquitoes, the hands are protected by thick gloves to prevent mosquito bites. The forearm applied with diluents should be inserted into the cage containing 50- 100 active host seeking female mosquitoes. The number of mosquitoes that land on and/or commence to probe the skin should be counted for a 30-second period. The volunteer should avoid movement of the arm during testing.

In order to continue the test further, the landing rate of mosquitoes should be at least ≥ 10 landings and/or probings in 30-second period. Now the control forearm should be carefully withdrawn and the same arm should be treated with the lowest dosage of repellent dissolved in 1 mL diluent and the arm should be allowed to dry for a minute. The treated arm should be placed inside the cage for another 30-second period and observed for mosquito landings and/or probings. The same procedure should be repeated for testing with each additional incremental repellent dose. Successive tests should be carried out one after the other without delay. The repellent dose at each test should be calculated as the sum of the doses applied to arrive at the cumulative dose for each test.

An example of successive doses applied to arrive at a cumulative dose in a sample experiment is given below (WHO/HTM/NTD/WHOPES/2009.4):

| Application sequence | Repellent solution Concentration to be applied in 1 mL (mg/mL) | Cumulative amount of repellent ₂ (mg/600 cm ² area) |
|----------------------|--|---|
| Left-arm control | Pre-treated with alcohol* only | - |
| Left-arm dose 1 | 1 | 1 |
| Left-arm dose 2 | 1 | 2 |
| Left-arm dose 3 | 2 | 4 |
| Left-arm dose 4 | 4 | 8 |
| Left-arm dose 5 | 8 | 16 |
| Right-arm control | Pre-treated with alcohol* only | - |

*Alcohol or the same diluents used in the preparation of the repellent solution.

If the landing and/or probing rate is very high to accurately count the landing mosquitoes, the counting may be restricted for 5 minutes and three such readings should be done. The total mosquito counts from these three readings (15 minutes) should be multiplied by two to estimate the landing rate that would take place in a 30-second period. If the mosquito landing and/or probing rate on the exposed forearm is <10 females in 30 seconds, testing of repellents should not be continued further. The above procedure should be followed consistently throughout the experiment.

For recording/counting the number of landing and/or probing, trained volunteer should be used. At the end of the experiment, 1 mL ethanol/diluent should be applied on the other (right)

forearm and allowed to dry for a minute. Now, this diluents treated forearm is to be inserted in the cage for 30-seconds. This is to verify that the landing/probing rate is approximately ≥ 10 per 30 seconds, as was observed at the beginning of the experiment. If the landing rate is < 10 females per 30 seconds, the results of this experiment should be discarded.

Protection (p) is expressed as a proportion of the number of mosquito landings and/or probings on the treated arm (T) in relation to the number of landings and/or probing on the control arm (C) of the same individual:

$$p = 1 - (T / C) = (C - T) / C$$

where C is the average of the landings/probings on the two untreated arms (the diluent-applied test arm before repellent treatment and the other arm at the end of the experiment). Data are analysed using probit-plane regression analysis from which the ED₅₀ and ED_{99.9} and their confidence limits can be estimated.

(b) Protection time

The complete protection time (CPT) of a repellent can be determined in two ways. First, the ED_{99.9} dose of candidate repellent should be estimated using the procedures described in section 2.4.1.3 (a). Following which, one mL of the candidate repellent is then tested at the ED_{99.9} dose against 1 mL of the standard 20% ethanolic deet (positive control). Otherwise, 1 mL of the 20% ethanolic deet solution can be tested and compared with the same quantity (weight/weight) of the candidate repellent on the other arm. In both of these experiments, repellent formulations are applied to $\approx 600 \text{ cm}^2$ surface area of the forearm between the wrist and elbow region.

Mosquitoes are held in two separate mosquito cages (size: 35–40 cm per side) for the experiment. One cage should be designated for testing the candidate repellent and the other one for testing the positive control (ethanolic deet). In each cage, 200–250 non-blood-fed active host-seeking females are released. Prior to the start of experiment, the hands should be protected by thick gloves to prevent mosquito bites. During the testing, the volunteer should avoid the movement of the arm(s).

At the start of experiment, the readiness of mosquitoes to land and/or probe must be assessed by placing an untreated (alcohol- or diluents treated) arm into a cage for 30 seconds and the level of landing rate is recorded. In the second cage, the same procedure is repeated using the other arm to assess the landing rate. In both cases, the level of landing/ probing rate should be ≥ 10 per 30 seconds. If this level of landing / probing is not observed in either cage, the experiment should be discarded.

In the beginning of the experiment, 1 mL of the candidate repellent prepared in alcohol/diluent solution is applied to one arm and allowed for drying for one minute. On the other arm, 1 mL of the standard deet solution is applied and allowed for drying. Now the repellent-treated arm should be inserted into the appropriate cage containing active host-seeking female mosquitoes and exposed for 3 minutes to determine landing and/or probing activity.

Subsequently, the deet-applied arm should be placed inside the other cage and exposed for 3 minutes to determine landing and/or probing activity. This procedure should be repeated at every 30 or 60-minute intervals. Exposure timings should be constant throughout the experiment.

The test for a particular repellent dose will come to an end when the incidence/occurrence of one landing and/or probing is observed in a 3-minute test interval. The number of minutes elapsed between the time of repellent application and the first mosquito landing and/or probing is calculated to determine complete protection time (CPT). Sufficient number of volunteers should be employed in the test to allow for statistical analysis. The Kaplan–Meier Survival Function test can be used to estimate the median CPT and its confidence intervals.

The dose of a repellent providing at least 6–8 h of protection in mosquito cage experiments may be considered to be an ideal compound for use in field as repellent for field evaluation. Data should be recorded in the format given in Table 32.

Table 32. Laboratory evaluation of repellents on humans (Cage method)

Name of the repellent..... Date..... Time
 Temp..... Relative humidity..... Dose of application.....

| Replicate number* | Time of first bite | Protection time | Percent protection |
|------------------------------------|--------------------|-----------------|--------------------|
| Dose 1 | | | |
| Dose 2 | | | |
| Dose 3 | | | |
| Dose 4 | | | |
| Positive control | | | |
| Negative control | | | |
| * Separate row for each replicate. | | | |

2.4.1.4. Human safety

A semi-structured questionnaire should be used to record the perceptions of human volunteers about the positive and/or negative side effects of the repellents (Annexure 3).

2.4.2. Field evaluation

Field trials are carried out to confirm the results of laboratory testing in order to estimate the efficacy, persistence and optimum application dose, of a repellent material, in terms of repellency and protection time, against the natural population of mosquito vectors and/or pest species in different ecological settings. The effective dose determined in the laboratory should be used in the field evaluation. Use of known repellent compound is optional. At least two field tests should be undertaken, one each in different ecological settings representing the habitat of target mosquito species where the human exposure takes place.

The field trials should be carried out on human volunteers. Selection of human volunteers for the field evaluation should be random without any bias. Prior to engagement, informed consent should be obtained from each volunteer for his participation in the evaluation (Annexure 4). The volunteers should be selected from the same village where the test will be conducted, so that they will not be exposed to unusual risk of infection. Wherever possible, the volunteers should be protected by chemoprophylaxis and/or vaccination appropriately.

Positive control (treated with DEET) and negative control (treated only with alcohol/diluents) should be included for assessing the repellency of the candidate repellent. Five volunteers, two for test repellent, two for positive control and one for negative control should be employed for the evaluation). Human volunteers should be placed at least 10 metres away from each other. Mosquitoes landing on the volunteers particularly on one or more bare limbs (knee to ankle; elbow to wrist), should be collected from dusk to dawn. Collections at different hours of night are held separately in test tubes and labelled. The mosquitoes are brought to laboratory and identified to species level. Insect collectors should be rotated every four hours to avoid slackness and bias. Both complete protection and percent repellency should be determined as described in laboratory evaluation (Section 2.4.1.3). Data should be recorded in the format given in Table 33.

Table 33. Mosquito landing collections

Name of the repellent.....Date..... Time..... Temp.....

Relative humidity.....Repellent and concentration.....Species.....

| Time (Hrs) | Number of bites | | | Percent protection | Average protection time |
|------------|-----------------|--------|--|--------------------|-------------------------|
| | Test 1 | Test 2 | Positive control 1 Positive control 2 Negative control | | |
| 1800 | | | | | |
| 1900 | | | | | |
| 2000 | | | | | |
| 2100 | | | | | |
| 2200 | | | | | |
| 2300 | | | | | |
| 2400 | | | | | |
| 0100 | | | | | |
| 0200 | | | | | |
| 0300 | | | | | |
| 0400 | | | | | |
| 0500 | | | | | |
| 0600 | | | | | |
| Total | | | | | |

Plants used for making mosquito repellents



Citronella (*Pelargonium citrosum*)



Catnip (*Nepeta cataria*)



Eucalyptus (*Eucalyptus globulus*)

2.5. Larvicides

Larvicides are used for vector control under disease control programmes. Larvicides include chemical larvicides, bio-larvicides and insect growth regulators. Evaluation of larvicides is carried out for both WHOPEs passed and new insecticides. New insecticides are evaluated in 3 Phases - I, II and III, while for WHOPEs passed insecticides only Phase II and III trials are conducted.

2.5.1. Chemical Larvicides

These compounds are generally nerve poisons and inflict mortality of mosquitoes at immature stages.

2.5.1.1. Phase I: Laboratory studies

2.5.1.1.1. Duration: 3 months.

2.5.1.1.2. Objectives

- to establish dose-response relationships of the larvicide against the target vector species,
- to determine LC₅₀ and LC₉₀,
- to establish a diagnostic dosage to discriminate between resistant and susceptible populations and
- to assess cross-resistance to the commonly used insecticides

2.5.1.1.3. Preparation of stock solutions and test concentrations

Since the technical grade insecticides are normally insoluble in water, stock solutions are prepared by dissolving the insecticide material in organic solvents (acetone or ethanol). For testing a formulated insecticide product, stock solution (1%) and subsequently, the serial dilutions are prepared using distilled water.

To prepare 20 ml of 1% stock solution, 200 mg of the technical grade material is dissolved in 20 ml solvent. The solution should be stored in a all glass screw cap vial with bakelite screw cap covered with aluminum foil. Complete dissolving or dispersion of the material in the solvent should be ensured by vigorous shaking. Serial dilutions of the stock solutions (ten-fold) are made in ethanol or other solvents (2 ml stock solution to 18 ml solvent). To obtain the test concentrations, 0.1 – 1.0 ml (100-1000 µl) of the appropriate dilution is added to 100 ml or 200 ml chlorine-free tap water or distilled water (Table 34). As given in the Table 34, aliquots of dilutions added should be adjusted for obtaining other volumes of test water. When a series of concentrations are prepared, the lowest concentration should be made first. Pipettes with disposable tips are preferably used to transfer small volumes of dilutions to test cups.

For testing a formulated insecticide product, stock solution (1%) and subsequently, the serial dilutions are prepared using distilled water following the same procedure given above.

Table 34. Aliquots of various strength solutions added to 100 ml water to yield final concentration (WHO/CDS/WHOPES/GCDPP/2005-13)

| | Initial solution | | Aliquot (ml)* | Final concentration |
|---------|------------------|--|---------------|---------------------|
| % | PPM | | | (PPM) in 100 ml |
| 1.0 | 10 000.0 | | 1.0 | 100.0 |
| | | | 0.5 | 50.0 |
| | | | 0.1 | 10.0 |
| 0.1 | 1000.0 | | 1.0 | 10.0 |
| | | | 0.5 | 5.0 |
| | | | 0.1 | 1.0 |
| 0.01 | 100.0 | | 1.0 | 1.0 |
| | | | 0.5 | 0.5 |
| | | | 0.1 | 0.1 |
| 0.001 | 10.0 | | 1.0 | 0.1 |
| | | | 0.5 | 0.05 |
| | | | 0.1 | 0.01 |
| 0.0001 | 1.0 | | 1.0 | 0.01 |
| | | | 0.5 | 0.005 |
| | | | 0.1 | 0.001 |
| 0.00001 | 0.1 | | 1.0 | 0.001 |
| | | | 0.5 | 0.0005 |
| | | | 0.1 | 0.0001 |

*For 200 ml double the volume of aliquots

2.5.1.1.4. Laboratory bioassays

Determination of LC₅₀ and LC₉₀ of larvicides is done on known age laboratory colonized larvae or F₁ larvae of the field collected adult mosquitoes. Bioassays are done following the WHO procedure (WHO/CDS/WHOPES/GCDPP/2005-13). The highest test concentration should not generally exceed 1 ppm or 1 mg/litre.

In laboratory bioassays, the activity range of the test material is determined first by exposing early IV instar larvae of the target mosquito species to a wide range of test concentrations and a control (no insecticide). Based on the larval mortality obtained with the wide range of concentrations, a narrow range (of 4-5 concentrations, causing mortalities between 10% and 95% at 24 h or 48 h) is used to determine LC₅₀ and LC₉₀.

Small disposable paper cups are used for the bioassays. The size of the cup should be in such a way that after filling with 100-200 ml of water, the depth of the water in the cups should be between 5 cm and 10 cm, as deeper levels may cause undue mortality. To each cup, 25 early IV instar larvae are transferred carefully with a small ring net or strainer.

The target dosages, starting with the lowest concentration, are obtained by adding appropriate volume of dilution (Table 34) to 100 ml or 200 ml water in the cups. For each concentration, four to five replicates are set up with parallel controls (keeping equal number of replicates) in chlorine free tap water. One ml of ethanol/acetone is added to each control replicate. Bioassays should be repeated for each concentration at least three times on different days, using freshly prepared stock solution and different batches of larvae each time. Since the exposure period is 24 hours no larval food is required. However, food (finely ground dog biscuit and yeast powder, 40: 60) may be provided if the exposure period is extended to 48 hours and beyond. The test cups are maintained at a temperature range of 25-28°C and at a photoperiod of 12 h light and 12 h dark period (12L:12D).

After the exposure period of 24 hours, % mortality is calculated counting dead and moribund larvae in the test replicates. Moribund larvae are those that are incapable of rising to the surface or not showing the characteristic diving action when the water is disturbed (WHO, 2005). The data are recorded as per the Table 35 (where, LC₅₀, LC₉₀ and LC_{99.9} values and the outcome of slope and heterogeneity analysis are also recorded).

If more than 10% larvae pupates or when more than 20% larval mortality occurs in the controls, the experiment should be discarded and repeated. If the larval mortality in control is between 5% and 20%, the treated mortality should be corrected according to the Abbott's formula:

$$\text{Corrected mortality (\%)} = \frac{X - Y}{X} \times 100$$

Where, X = Percentage survived in the control (untreated) and
Y = Percentage survived in the treated

2.5.1.1.5. Data analysis

For determination of LC₅₀, LC₉₀ and LC_{99.9}, data from all replicates should be pooled. The LC₅₀ and LC₉₀ are calculated from a log dosage-probit mortality regression line using a computer software programme or using a log-probit paper.

2.5.1.1.6. Determination of diagnostic concentration

Diagnostic dose is determined by multiplying the upper fiducial limit of LC_{99.9} with a factor of 2 for routine susceptibility test.

2.5.1.1.7. Cross-resistance assessment

To assess the cross-resistance to other insecticides currently in use in the programme, bioassays should be done using the diagnostic dosage of the test larvicide and of other larvicides.

2.5.1.2. Phase II: Small-scale field trial (multi-centric)

The larvicides that show promising activity in laboratory evaluation (Phase I) are considered for small-scale field testing (Phase II). The dosages for Phase II trials are calculated based on LC_{99.9} values determined under Phase I trial. The application dosages will be determined by multiplying the observed LC_{99.9} value with a factor of 2 and above so as to obtain at least a range of 3-5 dosages for small scale testing. The evaluation is to be done at least in three sites.

2.5.1.2.1. Duration: 6 months

2.5.1.2.2. Objectives

- To determine the efficacy and residual activity of the larvicide/ formulations against target vector species breeding in clear and polluted water habitats
- To determine the optimum field application dosage for Phase III trial
- To record qualitative observations on the non-target organisms, especially predators, cohabitating with mosquito larvae

2.5.1.2.3. Trial in natural breeding habitats

The field efficacy of the larvicide is tested in natural breeding habitats of the target species. Selection of habitats for the testing is done in such a way that all the major types (of habitats) are represented. For *Anopheles* species, cement tanks, drums, garden pits, pools, rice plots, river/ stream bed pools, water fountains and disused wells; for *Culex* species stagnant drains, cesspits, cesspools and disused wells and for *Aedes* spp. cement tanks, drums, peri-domestic water storage containers and water fountains are best suited. A minimum of three replicates of each type of habitat should be randomly selected for each dosage of the larvicide/ formulation, with an equal number of controls. The size of the habitat is recorded, taking in to account of surface area and depth. As far as possible, the habitats selected should be similar and comparable in terms of vectors prevalence and their density. Each of the confined breeding sources or containers can be considered as a discrete habitat or replicate. Habitats such as drains and canals may be divided into sectors of 10 m length and replicated for treatment and control.

Prior to application of larvicidal formulation, density of larvae and pupae should be monitored for a week at least on two occasions. Breeding habitats from each type with comparable pre-treatment densities should be allotted equally to treatment and control groups.

Density of larvae and pupae in the selected habitats is recorded prior to treatment by taking samples using a standard dipper (9 cm diameter with 300 ml capacity) (dipper method) for pits, ponds, tanks, drains, drums etc., or a bucket (3 litre capacity) for wells. Number of samples to

be taken from each habitat is decided based on the size of the habitat. For small habitats such as drums, pits, pools, tanks and wells, 3 to 5 dipper samples per habitat, while for stagnant drains, sampling at a distance of 3-5 m are recommended. The larval instars and pupae collected from each dipper sample are counted and recorded stage-wise and returned to the habitats. While monitoring the density of larvae and pupae observation is made on the presence of non-target organisms particularly the predators of mosquito larvae in the test habitats.

About 3-5 dosages of the larvicide should be applied to the breeding habitats. Formulations such as emulsifiable concentrate, suspension concentrate, liquids etc. should be applied to the habitats using a knapsack sprayer/ hand compression sprayer which should be calibrated prior to use and the rate of application be expressed per unit area.

The following formula is used to determine the application rate:

$$\text{Rate of application (ml/m}^2\text{)} = \frac{\text{Flow rate (ml/min)}}{\text{Width of swath (m) x walking speed (m/min)}}$$

The required concentration of larvicide suspension is calculated as follows:

$$\text{Concentration of larvicide (Emulsion/suspension)} = \frac{\text{Dosage to be applied (g/m}^2\text{)}}{\text{Application rate (ml/m}^2\text{)}} \times 100$$

Other formulations such as granules, pellets, tablets and briquettes can be manually broadcast or thrown in the water.

After the treatment, immature density (all stages) is monitored on day 1, 2, 3 and 7 post-treatment and then weekly until the density of IV instar larvae in the treated habitats reaches a level comparable to that in the control. To assess the impact of the larvicidal formulation on the non-target organisms, observation should also be made on their presence or absence during the post-treatment period to compare with the observation recorded before the treatment. Temperature and pH of habitat water should be recorded on each day of observation.

Data are recorded on the form as given in Table 36.

2.5.1.2.4. Data analysis

The mean number of pupae and larvae collected per dip is calculated for each sampling day and for each replicate. The reduction of density of larvae and pupae on post-treatment days will be estimated by comparing the pre- and post-treatment densities in the treated habitats with the corresponding densities in the untreated habitats using Mulla's formula.

$$\% \text{ Reduction} = 100 - \frac{C_1 \times T_2}{T_1 \times C_2} \times 100$$

Where,

- C₁ = Pre-treatment immature density in control habitats
- C₂ = Post-treatment immature density in control habitats
- T₁ = Pre-treatment immature density in treated habitats
- T₂ = Post-treatment immature density in treated habitats

The differences between the dosages can be compared using two-way ANOVA with dosage and day as the main factors after transforming percentage reduction (of immature density) to arcsine values. The interaction effect of dosage and day is used to compare the effect of treatment over days. Pair wise comparison of dosages is done using the pos-hoc test based on least significant difference (LSD). The mean arcsine values should be back transformed to percentage values for further interpretations. The effective duration (the post-treatment day up to which the lower limit of the 95% CI for the mean % reduction of density will be >80%) of each treatment/ dosage will be compared between the dosages to select the optimum application dosage (i.e. the lowest dosage that produces the maximum effective duration) for the Phase III trial.

As the optimum field application dosage will differ between clean and polluted water habitats, trials should be separately undertaken in these habitats to determine the optimum field application dosage.

2.5.1.2.5. Trial in simulated field condition

Testing is done in simulated conditions against the mosquito species that breed in domestic and peri-domestic clean water habitats. Trials can be carried out in containers (drums, jars, buckets, tubs, etc.). Against *Cx. quinquefasciatus*, testing is not done in simulated field condition.

The efficacy of the larvicidal formulations is tested against laboratory reared *Aedes aegypti*, *Ae. albopictus* and *Anopheles stephensi* larvae under simulated field conditions. Cement tubs with 100 lit or 200 lit capacity (used commonly by households) are preferably used for the trial. The diameter of the tubs at the water surface should be 75 cm.

Prior to testing, the cement tubs are decontaminated by filling them fully with water and setting them open in the sun for a week or two. The tubs are then emptied, scrubbed, rinsed thoroughly with water and dried for a day or two. The tubs are placed under a shed having only a roof and open on all sides, simulating the field condition. The placement of the tubs is configured in a block design form to equally distribute positional effects. The tubs are filled with domestic tap water (100 lit or 200 lit). The tubs are screened with nylon mesh to prevent egg laying by other mosquitoes or insects and to protect the water from falling debris.

Two regimens of water can be used: In the first regimen, tubs are kept full for the duration of the experiment without removing the water; and in the second regimen, half of the water in the tubs is removed and replenished weekly with fresh tap water to simulate water use conditions. The two regimens of water are used for control as well as treatment.

To assess the efficacy, a batch of 50-100 laboratory-reared early fourth instar larvae are released into each cement tub or replicate. To each tub, 0.5 gm ground up larval food is provided initially before adding first cohort of larvae and weekly thereafter. After 2-3 h of larval acclimatization, the tubs are treated with the larvicide/ formulation at 3-5 dosages in a randomized manner. In each water regimen, a minimum of four replicates of each dosage and four controls should be used. The water level in the tubs must be sustained.

All the containers are examined after 48 h of treatment and live larvae are counted to score post-treatment larval mortality. To test residual activity, the treatments are challenged with new cohorts of larvae (early third instar) of the same mosquito species weekly and larval food is provided on alternate days or weekly. Larval and pupal survival is assessed 48 h post-treatment. Data are recorded on the form as shown in Table 36.

The evaluation is continued until there is no statistically significant residual activity in terms of larval and pupal mortality in the treated habitats compared to the untreated controls. Temperature and pH of the habitat water should be recorded on the days of evaluation.

2.5.1.2.6. Data analysis:

Efficacy and residual activity of the larvicidal formulation at different dosages are determined from the post-treatment counts of live larvae and pupae in treated and control replicates as compared to the pre-treatment counts. The criteria for determining the level of effectiveness of a candidate larvicide should be >80% reduction in the pre-treatment counts (% reduction is calculated using the Mulla's formula). For analysis of data, the method described under section 2.5.1.2.4 can be used. Yet, since known number of larvae are released (the denominator) under simulated trials, a probit or logistic regression analysis will be more suitable.

The number of live, dead and moribund larvae and pupae from all replicates of each dosage on each day of observation should be pooled for calculating percentage mortality. Logistic or probit regression of the percentage mortality on dosages and number of post-treatment days are used to determine the effective duration (the post-treatment day up to which at least 80% mortality (and its 95% CI) (the desired level of control) is achieved for a given dosage.

The effective duration of the dosages tested under the Phase II trial in natural or simulated habitats will be compared and the lowest dosage that produces the maximum effective duration will be selected as the optimum field application dosage for the Phase III trial.

2.5.1.3. Phase III: Large-scale field trial (multi-centric)

In this phase, the larvicide is applied to the natural breeding habitats of the target mosquito species at the optimum field application dosage(s) selected in the small-scale field trials using appropriate application equipment, depending on the formulation. The large scale field trial should be conducted at least in three different eco-epidemiological settings.

2.5.1.3.1. Duration

Evaluation should be carried out for a period of one year covering all seasons.

2.5.1.3.2. Objectives

- To confirm the efficacy of the larvicidal formulation applied at the selected optimum field application dosage against the target mosquito species in natural breeding habitats
- To confirm residual activity and application intervals in clean and polluted habitats
- To record observations on the ease of application and dispersal of the insecticide
- To record residents' / community acceptance
- To document any perceived side-effects on operators and
- To observe the treatment effect on non-target organisms prevalent in the trial areas.

2.5.1.3.3. Selection of study area

A locality of one square kilometer of urban/ rural area is selected for treatment and a locality of similar type and size for control. All types of breeding habitats are surveyed to ascertain the breeding of the target species in order to decide the suitability of the locality for the trial. A selected locality should have a minimum of 25% larval habitat positivity for the target mosquito species. As in the case of small-scale field trials, each of the confined breeding habitats can be considered as a discrete habitat or replicate. Habitats such as drains and canals may be divided into sectors of 10 m length and replicated for treatment and control.

2.5.1.3.4. Assessment of density prior to treatment

Prior to treatment, density of larvae and pupae is assessed in treatment and control habitats on at least two occasions during a week. The immature density is measured in different types of habitats using appropriate sampling devices (as given in Section 2.5.1.2.3., 'small-scale field trials in natural breeding habitats').

2.5.1.3.5. Application of larvicidal formulation

In the selected locality, all breeding habitats should be treated at the optimum field application dosage determined in Phase II trial. In small-scale trial, if there is a wide variation between optimum dosages for each type of habitat, the specific optimum dosage should be applied to each type of habitat. Alternatively, the optimum dosage for the major larval habitat of the target species in the area can be used for other habitats. The habitats will be re-treated at intervals depending on the residual activity determined in phase II trial.

2.5.1.3.6. Assessment of density after treatment

The density of larvae and pupae is assessed by taking fixed number of samples from the treated and control habitats at 24 and 48 h post-treatment and thereafter at weekly intervals. Sampling procedures are similar to those followed for small-scale field trials in natural breeding habitats. Data should be recorded on the prescribed form (Tables 36). Observations will be made for a

minimum of three treatments for each trial. The trials should be repeated at least three times in different seasons.

2.5.1.3.7. Impact on adult density

The impact of the larvicidal treatment can also be assessed by measuring the density of adult mosquitoes resting indoors (human dwellings/ animal sheds) using hand catch method. At least 6-9 dwellings selected randomly will be used for estimating the adult density per man-hour in the treated and control areas. This may provide information on the trend in the reduction of adult population of target mosquito species due to the effect of the larvicidal application.

2.5.1.3.8. Effect on non-target organisms

For assessing the impact of the larvicidal application on non-target organisms that co-habitat with mosquito immature, their density can be monitored while sampling mosquito larvae and pupae during the large scale field trial. Larvivorous fish, snails, mayfly naiads, dragonfly naiads, copepods and aquatic beetles are some of the common non-target organisms that co-habitat with mosquito larvae.

2.5.1.3.9. Community acceptance

Information on ease of handling, application and storage of the larvicidal formulation should be collected and recorded. The effect of the larvicidal formulation on various parts of the application equipment (such as nozzle tips and gaskets, rotors, blowers, etc.,) should be collected and recorded to ensure a proper functioning of the equipment.

Acceptability of the residents of the area to the larvicide treatments, particularly in domestic and peri-domestic breeding habitats should also be recorded.

2.5.1.3.10. Data analysis

The mean number of pupae and larvae collected per dip is calculated for each sampling day and for each replicate. The statistical analysis described in section 2.5.1.2.4. will be followed to confirm the residual efficacy and frequency of application.

2.5.2. Bacterial Larvicides

Bacillus thuringiensis var *israelensis* is the only bacterial agent currently used in the vector control programme. It is a gram positive spore forming *Bacillus* that produces crystal toxins during sporulation. The crystal toxin contains the mosquito larvicidal toxin called delta-endotoxin which is thermostable up to 45 °C. This bacterium is lethal to mosquitoes as well as black flies. It has been found to have very high toxicity against *Culex*, *Anopheles* and *Aedes* mosquitoes in different habitats. The endotoxin of this bacterium is found to be safe.

2.5.2.1. Phase I: Laboratory studies

2.5.2.1.1. Duration: 3 months

2.5.2.1.2. Objectives

- to establish dose-response relationships of the given bacterial larvicide(s) against the target vector species,
- To determine LC₅₀ and LC₉₀ and dosages for Phase II trial

2.5.2.1.3. Preparation of stock solution and test concentrations

For conducting laboratory evaluation of the candidate biolarvicide, the first step will be to prepare a stock solution, normally 1%. To prepare 1% stock solution, 200 mg of solid or powder product is weighed and placed in a vial or flask (30 ml capacity) and 20 ml of distilled water is added to it. The contents are thoroughly homogenized using a serrated glass pestle/magnetic stirrer. The homogenate can be placed in air tight glass vials and frozen for future bioassays. The frozen stock solution should be homogenized completely for a few minutes before serial dilutions are made.

The stock solution is then serially diluted (ten-fold) in distilled water. The required test concentrations are obtained by adding 0.1–1.0 ml (100-1000 µl) to the test cups containing 100 ml of chlorine free water (Table 34).

2.5.2.1.4. Laboratory bioassays:

The laboratory bioassay procedures for bacterial larvicides are the same as those for chemical larvicides (Please refer to Section 2.5.1.1.4). Mortality is scored at 24 hours post-treatment for *B. thuringiensis* subsp. *israelensis* and 48 hours for *B. sphaericus* by counting the live larvae remaining in the test cups. When the exposure period is 24 h, addition of larval food to the test cups is not required and if the exposure period is extended to 48 hour food may be added. The results are entered on the form (Table 35).

2.5.2.1.5. Data analysis

LC₅₀ and LC₉₀ of the candidate biolarvicide will be calculated from mortality regression lines by probit analysis. The dosages for Phase II trials are calculated based on LC_{99.9} values determined under Phase I trial.

2.5.2.2. Phase II: Small-scale field trial (multi-centric)

Bacterial larvicides that show promising activity in the laboratory studies (Phase I) are subjected to small-scale field testing (Phase II). The application dosages for the Phase II trial will be determined by multiplying the observed LC_{99.9} value with a factor of 2 and above so as to obtain at least a range of 3-5 dosages for small scale testing.

2.5.2.2.1. Duration: 6 months

2.5.2.2.2. Objectives

- To determine the efficacy and residual activity of the bacterial larvicides/ formulations against target vector species breeding in clear and polluted water habitats
- To determine the optimum field application dosage for Phase III trial
- To record qualitative observations on the non-target organisms, especially predators, cohabitating with mosquito larvae

2.5.2.2.3. Trial in natural breeding habitats

The small-scale dose determination trial procedures for bacterial larvicides are the same as those for chemical larvicides (Section 2.5.1.2.3)

2.5.2.2.4. Data analysis: Refer to Section 2.5.1.2.4.

2.5.2.2.5. Trial in simulated field condition

The trial procedures in simulated field conditions for bacterial larvicides are the same as those for chemical larvicides (Section 2.5.1.2.5)

2.5.2.2.6. Data analysis: Refer to Section 2.5.1.2.6.

2.5.2.3. Phase III: Large-scale field trial (multi-centric)

In this phase, the bacterial larvicide is applied to the natural breeding habitats of the target mosquito species at the optimum field application dosage(s) selected in the small-scale field trials using appropriate application equipment, depending on the formulation. The trials are to be conducted at least in three different eco-epidemiological settings.

2.5.2.3.1. Duration

Evaluation should be carried out for a period of one year covering all seasons.

2.5.2.3.2. Objectives

- To confirm the efficacy of the bacterial larvicide applied at the selected field application dosage(s) against the target mosquito species in natural breeding sites
- To confirm residual activity and application intervals
- To record observations on the ease of application and dispersal of the insecticide
- To observe community acceptance
- To record any perceived side-effects on operators and
- To observe the effect of the treatment on non-target organisms

2.5.2.3.3. Evaluation

Selection of study sites, assessment of density prior to treatment, application of bacterial larvicide, assessment of post-treatment density, impact on adult density, effect on non-target organisms, operators' and residents' acceptability and data analysis for phase III evaluation of bacterial larvicides are the same as those for chemical larvicides. Refer to Sections 2.5.1.3.3 to 2.5.1.3.10.

2.5.3. Insect Growth Regulators (IGRs)

IGR compounds are of two types: 1. Chitin synthesis inhibitors (e.g. diflubenzuron, novaluron affect the synthesis of chitin during moulting of different instars of larvae and adult emergence from pupae thereby causing mortality at larval/ pupal stages and inhibition of adult emergence. In addition, the chitin synthesis inhibitors produce morphological deformities/ abnormalities among immature as well as emerging adults. 2. Juvenile hormone mimics (e.g. pyriproxifen and methoprene) delay the molting process of larvae and pupae thereby increasing the inter-moulting period and preventing the larvae from developing into adult insects. The toxicity of both types of insect growth regulators (IGRs) on mosquito larvae is assessed on the basis of inhibition of adult emergence and it is expressed as inhibition emergence percentage (IE %).

IGRs, besides inhibiting adult emergence, reduce oviposition rate and cause sterility of the eggs thereby reducing the reproductive potential of the female mosquitoes.

2.5.3.1. Phase I: Laboratory studies

2.5.3.1.1. Duration: 3 months

2.5.3.1.2. Objectives

- to establish dose-response relationships of the given insect growth regulator(s) against the target vector species,
- to determine the concentration of IGR for 50% and 90% inhibition of adult emergence (IE₅₀ and IE₉₀);

2.5.3.1.3. Determination of IE₅₀ and IE₉₀

Determination of IE₅₀ and IE₉₀ of insect growth regulators is done on laboratory reared larvae of known age or F₁ larvae of the field collected adult mosquitoes. Bioassays are done following the WHO procedure (WHO/CDS/WHOPES/GCDPP/2005-13).

2.5.3.1.4. Preparation of stock solution or suspension

The preparation of stock solution/ suspension and subsequent serial dilutions, and bio-assay set ups are the same as for the chemical larvicides (Sections 2.5.1.1.3 and 2.5.1.1.4).

2.5.3.1.5. Laboratory bioassays

The chitin synthesis inhibitors and the JH analogues should be tested on early III instar larvae. Since the bioassay duration is longer for IGRs, larval food should be added to the treated and control replicates at two-day intervals until mortality counts are made. All the treated and control replicates should be covered with mosquito netting to prevent escaping of emerged adults. Mortality is scored at every alternate day until the complete emergence of adults. The treated and control replicates are maintained at 25-28°C and a photoperiod of 12L: 12D.

The impact of IGR is expressed as percent inhibition of adult emergence (IE %). The IE% is estimated based on the number of larvae that do not develop successfully into adults. For each concentration, the moribund and dead larvae and pupae, as well as adult mosquitoes that are not completely separated from the pupal cases, are considered as “affected” for estimating IE%. Counting of empty pupal cases (exuvia) will provide the actual number of successfully emerged adults. The experiment comes to an end when all the larvae or pupae in the controls have died or emerged as adults. Data are entered on a form (Table 37). Morphological deformities/ abnormalities, if any, of the moulting larvae and pupae or the emerging adults should be recorded.

2.5.3.1.6. Data analysis

For the analysis, the data obtained from all replicates of each dosage should be pooled. IE% (total or mean) is calculated on the basis of the number of third stage larvae initially exposed using the following formula:

$$IE (\%) = 100 - (T \times 100)/C,$$

Where T= percentage survival or emergence in treated batches and
C= percentage survival or emergence in the control.

As in the case of chemical larvicides/ biolarvicides, if adult emergence in the control groups is <80%, the test should be discarded and repeated. When the percentage emergence in the control is between 80% and 95%, the data from the treated groups should be corrected to the control using Abbot’s formula. The IE₅₀ and IE₉₀ values are estimated from a log dosage-probit mortality regression analysis using computer software programs or estimated from log-probit paper. The procedure for data analysis given in the Section 2.5.1.1.5 should be followed. The dosages for Phase II trial are determined by multiplying the upper fiducial limit of IE_{99.9} with a factor of 2 and above so as to get 3-5 dosages.

2.5.3.2. Phase II: Small-scale field trial (multi-centric)

Duration: 6 months

2.5.3.2.1. Trial in natural breeding sites

The effect of IGR is evaluated by monitoring percentage reduction of larval and pupal densities and % inhibition of adult emergence. The evaluation procedures for larvae and pupae are similar to those followed for Phase II evaluation of chemical larvicides in natural breeding sites (Refer to section 2.5.1.2).

Monitoring of adult emergence can be made directly in the field by floating sentinel emergence traps or by sampling and counting pupal skins in treated and untreated habitats (Table 38). Alternatively, adult emergence can also be monitored by collecting pupae (20-40 per replicate) along with water from the treated and untreated habitats, bringing them to the laboratory, placing them in holding cages and observing adult emergence. Any morphological abnormalities in emerging adults should be recorded.

In the case of field evaluation with emergence traps, the adult emergence data obtained in treated and untreated habitats during pre-and post-treatment period should be used for the calculation of IE% using the following formula:

$$IE (\%) = 100 = (C1/T1) \times (T2/C2) \times 100$$

Where,

- C1 = number of adults emerged in control habitats before treatment,
- C2 = number of adults emerged in control habitats at a given interval after treatment,
- T1 = number of adults emerged in treated habitats before treatment and
- T2 = number of adults emerged in treated habitats after treatment.

In laboratory assessment, data on adult emergence from the pupal samples collected from treated and untreated habitats are used to calculate the IE% using the following formula (See also Section 2.5.3.1.6.).

$$IE (\%) = (C-T/C) \times 100,$$

Where,

- C = percentage emerging or living in control habitats and
- T = percentage emerging or living in treated habitats.

2.5.3.2.1.1. Data analysis:

The mean larval and pupal density (number per dip) is calculated for each day of sampling and for each replicate. Subsequently, the pre- and post-treatment densities in the treated and untreated habitats will be used to calculate the percentage reduction of larval and pupal densities or the IE% on post-treatment days using Mulla's formula. The difference between the treatments/ dosages is compared using two way ANOVA. For other details the section 2.5.1.2.4 may be referred to.

As the optimum field application dosage will differ between clean and polluted water habitats, trials should be separately undertaken in these habitats to determine the optimum field application dosage.

2.5.3.2.2. Trial in simulated field condition

The study design and sampling procedures of the simulated field trial of IGRs are similar to those followed for evaluation of chemical larvicides (Refer to section 2.5.1.2.5) except the following aspects.

After the treatment with IGR, the number of live larvae and pupae is counted at 2 days interval. In addition to larval/ pupal counting, pupal exuvia should be counted in both treated and untreated habitats as presence of pupal exuvia gives an accurate measurement of adult emergence. To test residual activity, the treatments are challenged with new cohorts of larvae (third instar) of the same mosquito species weekly and larval food is added on alternate days or weekly. Counting of live larvae and pupae, and pupal skins is done at every 2 days after the addition. Alternatively, the pupae are removed from the treated and untreated containers on alternate days and kept in cups with water from the respective containers, then placed in cages and adult emergence is recorded. Adults which are not fully freed from pupal skins should be counted dead. Temperature and pH of the habitat water should be recorded throughout the evaluation. The evaluation process continues until no mortality or inhibition of adult emergence is observed. Data are recorded on the form in Table 38.

2.5.3.2.2.1. Data analysis:

In laboratory assessment of adult emergence from the pupal samples collected from treated and untreated containers, IE% is calculated using the following formula, (see also Section 2.1.1.3);

$$IE (\%) = (C-T/C) \times 100,$$

Where,

C = percentage emerging or living in control habitats and

T = percentage emerging or living in treated habitats.

The mean number of pupae and larvae collected per dip is calculated for each sampling day and for each replicate. The reduction of larval and pupal densities or the IE% on post-treatment days will be estimated by comparing the pre- and post-treatment densities in the treated habitats with the corresponding densities in the untreated habitats using Mulla's formula. The method given in Section 2.5.1.2.4 should also be used to analyze data collected under simulated trials. However, since the denominator is known for simulated trials, a probit or logistic regression analysis is more suitable than ANOVA and is described in section 2.5.1.2.6.

The effective duration (the post-treatment day up to which the lower limit of the 95% CI for the mean % reduction of density will be $\geq 80\%$) of each treatment/ dosage will be compared between the dosages tested in the natural or simulated habitats to select the optimum application dosage (i.e. the lowest dosage that produces the maximum effective duration) for the Phase III trial.

2.5.3.3. Phase III: Large-scale field trial (Multi-centric)

In this phase, the IGR is applied to the natural breeding habitats of the target mosquito species at the optimum field application dosage(s) selected in the small-scale field trials using appropriate application equipment, depending on the formulation. The trials are to be conducted at least in three different eco-epidemiological settings.

2.5.3.3.1. Duration

Evaluation should be carried out for a period of one year covering all seasons.

2.5.3.3.2. Objectives

- To confirm the efficacy of the IGR applied at the selected field application dosage(s) against the target mosquito species in natural breeding sites To confirm residual activity and application intervals separately for clear/ polluted water habitats
- To record observations on the ease of application and dispersal of the IGR
- To observe residents' / community acceptability
- To record any perceived side-effects on operators and
- To observe the effect of the treatment of IGR on non-target organisms

2.5.3.3.3. Evaluation

Selection of study sites, assessment of density prior to treatment, application of IGR, assessment of post-treatment density, impact on adult density, operators' and residents' acceptability and data analysis for phase III evaluation of IGR are the same as those for chemical larvicides. Refer to Sections 2.5.1.3.3 to 2.5.1.3.10.

Adult emergence can be monitored directly in the field by floating sentinel emergence traps in treated and untreated habitats or by collecting pupal samples (20-40 per replicate) along with water from treated and untreated habitats and observing for adult emergence (as described under section 2.5.3.2.1., "small-scale field trials with natural population).

2.5.3.3.4. Effect on non-target organisms:

The IGRs affect moulting process of insects either by inhibiting chitin synthesis or prolonging the inter-moulting period that result in inhibition of adult emergence. In clear water habitats, the non-target organisms likely to be affected are only the aquatic stages of insects. In large clear water collections such as ponds, small lakes etc. in addition to aquatic insects, crabs, prawns (crustaceans), etc. are to be considered to study the effect of IGR on these organisms. In polluted habitats, the effect of IGR should be assessed against aquatic stages of insects, if present.

Table 35: Laboratory evaluation of the efficacy of larvicides against mosquito larvae

Experiment No: _____

Investigator: _____

Treatment date: _____

Larvicide: _____

Test species: _____

Larval instar: _____

Temperature: _____

Lighting: _____

| Conc. (mg/L) | Replicates | Number exposed | 24 Hours | 48 Hours | % Mortality | Corrected % Mortality |
|-----------------|------------|----------------|---------------|---------------|-------------|-----------------------|
| | | | Dead/Moribund | Dead/Moribund | | |
| Control | 1 | | | | | |
| | 2 | | | | | |
| | 3 | | | | | |
| | 4 | | | | | |
| T1 | 1 | | | | | |
| | 2 | | | | | |
| | 3 | | | | | |
| | 4 | | | | | |
| T2 | 1 | | | | | |
| | 2 | | | | | |
| | 3 | | | | | |
| | 4 | | | | | |
| T3 | 1 | | | | | |
| | 2 | | | | | |
| | 3 | | | | | |
| | 4 | | | | | |
| T4 | 1 | | | | | |
| | 2 | | | | | |
| | 3 | | | | | |
| | 4 | | | | | |
| T5 | 1 | | | | | |
| | 2 | | | | | |
| | 3 | | | | | |
| | 4 | | | | | |

LC50 (CL 95%): _____

LC90 (CL 95%): _____

LC 99.9: _____

Slope: _____

Heterogeneity: _____

Table 37: Laboratory evaluation of the efficacy of insect growth regulators (IGRs) against mosquito larvae

Experiment No: _____ Investigator: _____ Treatment date: _____
 IGR: _____ Test species: _____ Larval instar: _____
 Temperature: _____ Lighting: _____ L: Larvae; P: Pupae; A: adults

| Conc. (mg/L) | Replicates | Number exposed | 24 Hours | | | 48 Hours | | | Grand Total | | | | | | % Mortality | Corrected % Mortality |
|-----------------|------------|-------------------|----------------|---|---|---------------|---|---|-------------|---|---|---------------|---|---|----------------|--------------------------|
| | | | Alive | | | Dead/Moribund | | | Alive | | | Dead/Moribund | | | | |
| | | | L | P | A | L | P | A | L | P | A | L | P | A | | |
| Control | 1 | | | | | | | | | | | | | | | |
| | 2 | | | | | | | | | | | | | | | |
| | 3 | | | | | | | | | | | | | | | |
| | 4 | | | | | | | | | | | | | | | |
| T1 | 1 | | | | | | | | | | | | | | | |
| | 2 | | | | | | | | | | | | | | | |
| | 3 | | | | | | | | | | | | | | | |
| | 4 | | | | | | | | | | | | | | | |
| T2 | 1 | | | | | | | | | | | | | | | |
| | 2 | | | | | | | | | | | | | | | |
| | 3 | | | | | | | | | | | | | | | |
| | 4 | | | | | | | | | | | | | | | |
| T3 | 1 | | | | | | | | | | | | | | | |
| | 2 | | | | | | | | | | | | | | | |
| | 3 | | | | | | | | | | | | | | | |
| | 4 | | | | | | | | | | | | | | | |
| T4 | 1 | | | | | | | | | | | | | | | |
| | 2 | | | | | | | | | | | | | | | |
| | 3 | | | | | | | | | | | | | | | |
| | 4 | | | | | | | | | | | | | | | |
| T5 | 1... | | | | | | | | | | | | | | | |
| LC50 (CL 95%): | | | LC90 (CL 95%): | | | | | | LC 99.9: | | | | | | | |
| Slope: | | | Heterogeneity: | | | | | | | | | | | | | |

Table 38: Field evaluation of insect growth regulators against mosquito larvae

Experiment No: _____ Locality: _____ Street: _____ Investigators: _____

Habitat type: _____ IGR formulation: _____ Species: _____ Treatment date: _____

| Date | Days Pre/post-treatment | Dosage/control | Rep. No. | Sample No. | Immature density | | | | | | | No. of adult Emerged (traps) | No. of pupal skins | No of NTOs | Water Temp | pH | Surface area |
|------|-------------------------|----------------|----------|------------|------------------|----|------|-----|----|--------|---|------------------------------|--------------------|------------|------------|----|--------------|
| | | | | | I | II | I+II | III | IV | III+IV | P | | | | | | |
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NTO: Non-target organism; Rep: Replicate

2.6. Monomolecular Films

The monomolecular films (MMF) of organic compounds can act as a larvicide by reducing the surface tension of the aqueous surface and subsequently killing the immature by interfering with spiracular opening at the water interface and preventing tracheal respiration. Because of this property this can be used as larval control measure. Monomolecular films are effective only on clean water surface.

2.6.1. Phase I: Laboratory study

2.6.1.1. Duration: 3 months.

2.6.1.2. Objective

- To assess the effective dosage and efficacy of MMF

2.6.1.3. Determination of the dosage for treatment and its efficacy

In laboratory trials monomolecular film should be tested against the four instars and pupae. Different doses (0.1 to 1.0 ml/m²) should be applied and tested starting from the lowest dosage. Effective dose is one, which forms a monomolecular layer over the entire surface of water, as determined in laboratory. Rectangular enamel trays (45 x 30 cm) or (90 x 60 cm) should be filled with known volume of water (2 to 5 litres, ensuring at least 5 cm water column) and MMF should be applied in 6 different doses in separate trays. The effective dose is the lowest dose that covers the entire surface of the water with an uninterrupted film. This can be ascertained by putting rice husk or coloured powder supplied by the manufacturer as indicator for spreading.

To determine the efficacy of the selected dose, 100 laboratory colonized I/II instar larvae and III/IV instar larvae and pupae should be released in individual trays with parallel control. For each dosage and each instar a minimum of 3 replicates should be set and mortality of larvae/pupae should be recorded after 24, 48, 72 h and up to 2 weeks or more. Data should be recorded in the format given in Table 39. In general, control of 1st to 4th instar mosquito larvae is relatively slower than control of pupae, and control of younger instars (1st to 3rd) is slower than 4th instars.

Table 39. Observations on the MMF (to be revised)

| No. exposed | Mortality after 24 h | Pupal /Adult emergence | | | | |
|---------------|-------------------------|------------------------|------|--------|--------|--------|
| | | 48 h | 72 h | 1 week | 2 week | 3 week |
| I/II Instar | | | | | | |
| III/IV Instar | | | | | | |
| Pupae | | | | | | |

2.6.2. Phase II: small-scale field trial

2.6.2.1. Duration: 6 months.

2.6.2.2. Objectives

- To evaluate the efficacy of MMF in different natural habitats or in simulated habitats
- To assess the residual activity of the MMFs in different breeding habitats of the target vector species
- To determine the optimum field application dosages (s) and for Phase III trial.

2.6.2.3. Trials in natural breeding habitats

Natural breeding habitats of the target species have to be selected for the evaluation. For *Anopheles* spp. cement tanks, drums, pits, pools, water fountains and disused wells; for *Cx. quinquefasciatus* stagnant drains, pits, pools and disused wells and for *Aedes* spp. tanks, drums, discarded tyres, peri-domestic water storage containers, coolers and water fountains are best suited. A minimum of 3 replicates should be randomly selected for each type of habitat and dose. Equal number of control should be maintained for comparison. Temperature, pH and water quality (polluted or clean) should be recorded.

Pre-treatment larval and pupal density should be monitored by dipping method using a standard dipper (300 ml capacity with 9 cm diameter) for pits, ponds, tanks, drains, drums, etc., and bucket (3-liter capacity) or well net for wells. In addition, the adult emergence can be monitored using emergence traps (for details refer section 2.5.1.2.4). Number of samples to be taken from each habitat should be decided on the basis of type and size of the habitat. For small habitats such as drums, pits, pools, tanks and wells, 3 to 5 dips per site, while for stagnant drain dips at a distance of 5 m are recommended. The larval instars and pupae collected from each dipper/bucket sample are counted stage wise and returned back to the habitats.

About three to five dosages of MMF formulation are used in small-scale field trials based on manufacturers label instructions or laboratory studies. The MMF should be applied to the breeding habitats using appropriate spray equipment (e.g., compression sprayer). Spreading action of the MMF formulation is checked by the movement of rice husk placed on the water surface before application. The rate of application is expressed per sq. m.

After the treatment, the larval and pupal density and adult emergence is monitored in treated and control larval habitats at 24, 48 and 72 h and later at weekly intervals until the density of IV instar larvae and pupae reaches a level comparable to that in control. Data are recorded in the format given in a Table 40.

The mean number of larvae or pupae collected per dip and mean number of adults emerged per trap were calculated for each sampling day and for each replicate. Percent reduction in early (I/ II stage), late (III/ IV stage) larval instars, pupae and adult emergence should be calculated using the Mulla's formula (refer section 2.5.1.2.4). The differences between the dosages can be compared by two-way ANOVA (refer section 2.5.1.2.4). Persistence of the MMF formulation in different breeding habitats of the target species is determined from the post-treatment density of larvae and pupae in treated and control sites as compared to the pre-treatment density. The

minimum dosage at which maximum reduction (>80%) is achieved for longer duration should be selected as optimum field application dosage for each habitat.

2.6.2.4. Trial in simulated field condition

These trials are conducted for the mosquito species breeding in domestic and peri-domestic habitats in clean water. Trials should be carried out in containers (drums, tanks, etc.). For *Cx. quinquefasciatus* field trials are not undertaken in simulated condition. For anophelines, cement tanks, each having a capacity of 100 litres filled with 40 to 50 litres of potable water with different concentrations of insecticides should be used. In each tank 1000 to 2000 first instar larvae of the target species should be released at weekly intervals until the completion of the experiment.

Trials for *Ae. aegypti* should be carried out in 10 to 20 litre capacity drums with 5–10 litres of potable water. 100 to 200 larvae of *Aedes* species should be introduced into the treated drums at weekly intervals until the completion of experiment. The tanks and drums should be covered with specially designed emergence traps (dome shaped) to score adult emergence and prevent oviposition by other mosquito species/insects.

Water level in the tanks/drums should be maintained and finely ground larval food should be added without disturbing the monomolecular film on the water surface until the completion of the experiment. Prior to treatment, larval and pupal and adult emergence should be determined. The live immature stages should be released back into the respective tanks/ drums.

The MMF formulation, at 3-5 selected dosages within the recommended range of doses should be applied using appropriate spray equipment. Each dose should be applied to a minimum of 4 replicates (tanks/drums). Equal number of controls should be maintained for comparison.

During post-treatment, larval and pupal density and adult emergence should be monitored in treated and control habitats at 24, 48, 72 h and subsequently every 2-3 days intervals until there is no statistically significant residual activity in terms of larval and pupal mortality in the treated habitats compared to untreated control. Initial and long-term efficacy should be assessed on the basis of the observed larval and pupal density and adult emergence. Data should be entered in the prescribed form.

Efficacy and residual activity of the MMF are determined from the post-treatment counts of larvae and pupae and adult emergence in treated and control replicates as compared to the pre-treatment counts. The criteria for determining the level of effectiveness of a candidate MMF formulation should be >80% reduction in the pre-treatment counts (% reduction is calculated using the Mulla's formula) (Table 41). The minimum dosage causing the maximum reduction (>80%) for a longer duration should be selected as the optimum field application dosage for each habitat.

2.6.3. Phase III: Large-scale field trial

In this phase, the MMF is applied to the natural breeding habitats of the target mosquito species at the optimum field application dosage(s) selected in the small-scale field trials. The large scale field trial should be conducted at least in three different eco-epidemiological settings.

2.6.3.1. Duration: Evaluation should be carried out for a period of one year covering all seasons

2.6.3.2. Objectives

- To confirm the efficacy of MMF against larvae/ pupae in a locality
- To confirm the residual activity and frequency of application of the MMF in natural habitats.
- To record the observations on the ease of application and dispersal of MMF.
- To record residents acceptance.
- To observe the treatment effect on non-target organisms co-habiting with the target mosquito vectors.

2.6.3.3. Study area

A locality of one square kilometre of urban/rural area should be selected for treatment and a locality of similar type and size for control. All the types of breeding habitats are surveyed to ascertain the breeding of target species in order to decide the suitability of the locality for the trial. A selected locality should have a minimum of 25 percent larval habitat positivity for the target mosquito species. As in the case of small-scale field trials, each of the confined breeding habitats can be considered as a discrete habitat or replicate. Habitats such as drains and canals may be divided into sectors of 10 m length and replicated for treatment and control.

Prior to treatment, density of larvae and pupae is assessed in treatment and control habitats on at least two occasions during a week. The immature density is measured in different types of habitats using appropriate sampling devices (as given under 'small-scale field trials in natural breeding habitats').

In the locality selected for treatment, all the breeding habitats should be treated with the MMF formulation at the optimum field application dosage(s) determined in phase II trial. The MMF formulation is applied using appropriate spray equipment. Spreading action of the MMF formulation is checked by the movement of rice husk placed on the water surface before application. The habitats will be re-treated either at weekly/ fortnightly/monthly intervals depending on the residual activity determined for the MMF formulation for each type of habitat in Phase II trial.

Post-treatment density of larvae and pupae is assessed by taking fixed number of samples from the treated and control habitats at 48 h post-treatment and thereafter at weekly intervals. Sampling procedures are similar to those followed for small-scale field trials in natural breeding habitats. Data should be recorded on the prescribed form. Observations will be made for a minimum of three treatments for each trial. The trials should be repeated at least three times in different seasons.

The impact of the MMF treatment can also be assessed by measuring the density of adult mosquitoes resting indoors (human dwellings/ animal sheds) using hand catch method (for details refer Section 2.5.1.3.7). This may provide information on the trend in the reduction of

adult population of target mosquito species due to the effect of the MMF application. For assessing the impact of the MMF application on non-target organisms that co-habitat with mosquito immature, their density can be monitored while sampling mosquito larvae and pupae during the large scale field trial. Information on ease of handling, application and storage of the the MMF formulation should be collected and recorded. Acceptability of the residents of the area to the MMF treatments, particularly in domestic and peri-domestic breeding habitats should also be recorded.

The mean number of pupae and larvae collected per dip is calculated for each sampling day and for each replicate. The statistical analysis described in section 2.5.1.2.4 will be followed to confirm the residual efficacy and frequency of application.

Table 40. Field evaluation of MMF formulation against *Anopheles/ Culex/ Aedes* larvae/ pupae in tested habitat (Type of habitat)

| Duration after Treatment | Mean no. of 5 dips | |
|--------------------------|------------------------|-----------------------------------|
| | I and II Instar larvae | III and IV instar larvae Pupae |
| 0 day | | |
| 1 day | | |
| 2 day | | |
| 3 day | | |
| 4 day | | |
| 1 week | | |
| 2 week | | |
| 2 week | | |
| ... | | |
| 8 week | | |

Table 41. Percentage reduction of larvae/ pupae in MMF formulation treated habitat (Type of habitat)

| Habitat | Dosage | Hours/ Day post-treatment | % reduction larvae | % reduction pupae | Total |
|---------|----------|---------------------------|--------------------|-------------------|-------|
| Pond | Dosage 1 | 0 day | | | |
| | | 1 day | | | |
| | | 2 day | | | |
| | | 3 day | | | |
| | | 4 day | | | |
| | | 1 week | | | |
| | | 2 week | | | |
| | | 3 week | | | |
| | | | | | |
| | | 8 week | | | |

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4. ANNEXURES

Annexure 1

GUIDELINES FOR DEVELOPMENT OF INFORMED CONSENT FORM (WHO/HTM/NTD/WHOPES/2009.2)

For: [name the group of individuals for whom this consent is written]

Name of principal investigator:

Name of organization:

Name of sponsor:

Name of proposal:

PART I: Information sheet

This sheet is a suggestion or an example that can be modified according to the national rules and guidelines.

1. Introduction

State briefly who you are, and explain to participants that you are inviting them to take part in research that you are doing.

2. Purpose of the research

Explain in lay terms why you are doing the research.

3. Type of research intervention

State briefly the type of intervention that will be undertaken.

4. Participant selection

State why this participant or household has been chosen for this research. The selection will ensure that equal opportunities are provided to everybody.

5. Voluntary participation

Indicate clearly that volunteers can choose to participate or not. State that they will still receive all the services they usually do whether they choose to participate or not.

6. Information on the test product [name of the test product]

Explain to the participant why you are testing a product. Provide as much information as is appropriate and understandable about the product, such as its manufacturer or location of manufacture, and the reason for its development. Explain the known experience with this product. Explain comprehensively, if any, all the known side-effects or toxicity of this product.

7. Description of the process, procedures and protocol

Describe or explain to the participant the exact procedures that will be followed on a step-by-step basis and the tests that will be done.

8. Duration

Include a statement about the time commitments of the research for the participant, including the duration of the research and follow-up.

9. Side-effects

Potential participants should be told if there are any known or anticipated side-effects and what will happen in the event of a side-effect or an unexpected event.

10. Risks

Explain and describe any possible or anticipated risks. Describe the level of care that will be available in the event that harm does occur, who will provide it and who will pay for it.

11. Discomforts

Explain and describe the type and source of any anticipated discomforts that are in addition to the side-effects and risks discussed above.

12. Benefits

Mention only those activities that will be actual benefits and not those to which they are entitled regardless of participation.

13. Incentives

State clearly what you will provide the participants with as a result of their participation. WHO does not encourage incentives. However, it recommends that reimbursements for expenses incurred as a result of participation in the research be provided.

14. Confidentiality

Explain how the research team will maintain the confidentiality of data, especially with respect to the information about the participant, which would otherwise be known only to the physician but would now be available to the entire research team.

15. Sharing the results

Where relevant, your plan for sharing the findings with the participants should be provided.

16. Right to refuse or withdraw

This is a reconfirmation that participation is voluntary and includes the right to withdraw.

17. Whom to contact

Provide the name and contact information of someone who is involved, informed and accessible (a local person who can actually be contacted). State also that the proposal has been approved, and how.

This proposal has been reviewed and approved by [name of the local ethical committee], whose task is to make sure that research participants are protected from harm. If you wish to find out more about the Local Ethical Committee, please contact [name, address and telephone number].

PART II: Certificate of Consent

This section can be written in the first person. It should include a few brief statements about the research and be followed by a statement similar to the one in bold below. If the participant is illiterate but gives oral consent, a witness must sign. A researcher or the person checking the informed consent must sign each consent form.

Print name of participant: _____

Signature of participant: _____

Date: _____

day / month / year

Annexure 2

Assessment of perceived benefits, side-effects and collateral benefits of indoor residual spraying

Date of sprayingDate of interview/discussion

1. Name of respondent: (Optional).....
 2. Age:.....
 3. Sex:.....
 4. Education status:.....
 5. Village name:.....
 6. Do you know that insects transmit diseases ?.....
 7. If you know, name the diseases
 8. Do you protect yourself and family against these diseases ?.....
 9. If so, how..... Indigenous.....Commercial
 10. Are you aware whether something was sprayed in your house?
If yes, when and why.....
 11. Generally how many people sleep in the sprayed rooms(s)?.....
 12. Do you sleep in sprayed room?
 13. How does it smell?.....
 14. Do the sleepers feel suffocated?.....
 15. Have you allowed spraying in all rooms?: If no, reasons
 16. Does the insecticide leave stains on walls?
 17. Any fear of poisoning:.....
 18. Observations/perceptions of the effect of insecticide
 - on mosquito bites
 - on bed bugs
 - on head lice
 - on body lice
 - on domestic animals
 - any other
 19. Do you agree to use insecticide spray in future? YES/NO
- Reasons

Signature or LTI of inhabitant

Signature of Interviewer

Place/Date:

(This format should be translated into respective local language(s) in the study area and provided to the householder and read to him. A copy of the signed consent form should be given to the householder).

Annexure 3

Human safety observations after insecticide exposure

(Medical Practitioner should fill this proforma)

Project Title:..... Institute:.....

Part A. Medical case history form

1. Spray man/Volunteer S.No.:.....
2. Name:.....
3. Age(yr):
4. Gender:.....
5. Occupation:
6. Address:.....
7. Past history:.....
 - a. Illness: Yes/No b. Poisoning: Yes/No c. Allergy: Yes/No
8. Exposure to pesticides (mention compound, duration of exposure etc.):
9. Family History:
 - a. Allergy: Yes/No b. Mental Illness: Yes/No c. Hemorrhagic disorders: Yes/No
10. Personal history
 - a. Protective clothing: Complete/ Partial/ None
 - b. Ablutions (washing/bathing/clothes changing): Good/ Fair/ Poor
 - c. Personal habits: Smoking/ Alcohol/ Other addictions
11. Weather conditions: Temperature: Min.....Max.....
Relative humidity (%): Min..... Max.....
12. Clinical profile (sign & symptoms) pre- and post-exposure:

| | |
|-------------------------|-------------------------|
| (a) Vital signs | Pre-exposure (/ /2000) |
| Pulse rate | |
| Respiratory rate/minute | |
| Depth of respiration | |
| Temperature oF | |
| Chest tightness | |
| (b) General | Pre-exposure |
| Weakness | |
| Fatigue | |
| Sleep | |
| Urination | |
| Sweating | |

(Contd.....)

| | | | | | |
|---|--------------|-----|------|------|------|
| (c) Gastro-intestinal | Pre-exposure | 1 h | 24 h | 48 h | 72 h |
| Nausea | | | | | |
| Vomiting | | | | | |
| Appetite | | | | | |
| Taste | | | | | |
| Abdomen pain | | | | | |
| Diarrhoea | | | | | |
| Siallorrhoea | | | | | |
| (d) Neuro-muscular | Pre-exposure | 1 h | 24 h | 48 h | 72 h |
| Headache | | | | | |
| Dizziness | | | | | |
| Irritability | | | | | |
| Pain | | | | | |
| Twitchings | | | | | |
| Tremors | | | | | |
| Convulsions | | | | | |
| Parasthesia | | | | | |
| Hallucinations | | | | | |
| Unconsciousness | | | | | |
| (e) Cardio-respiratory | Pre-exposure | 1 h | 24 h | 48 h | 72 h |
| Nasal discharge | | | | | |
| Wheeze | | | | | |
| Cough | | | | | |
| Expectoration | | | | | |
| Chest tightness | | | | | |
| Dyspnea | | | | | |
| Palpitation | | | | | |
| Heart conserveness | | | | | |
| Cyanosis | | | | | |
| Tachycardia | | | | | |
| (f) Eye | Pre-exposure | 1 h | 24 h | 48 h | 72 h |
| Miosis | | | | | |
| Lacrimation | | | | | |
| Double vision | | | | | |
| Blurred vision | | | | | |
| (g) Psychological | Pre-exposure | 1 h | 24 h | 48 h | 72 h |
| Temperament | | | | | |
| Judgement | | | | | |
| Nervousness | | | | | |
| Insomnia | | | | | |
| X = No; N = Normal; NAD = Nothing abnormal detected; Skin (Dermal reaction/Irritation/Allergic reaction): | | | | | |

13. Human toxicology proforma for liver and kidney function tests

Liver function tests

- 1. Serum bilirubin
- 2. SGO
- 3. SGPT
- 4. Serum alkaline phosphatase
- 5. Serum protein

Kidney function tests

- 1. Blood urea
- 2. Serum creatinine

Signature of Medical Officer/Physician

Date: (Seal)

Place:

Part B. Nerve conduction studies in spraymen

- 1. Time of recording and sample size—Study should be on at least 5 spraymen exposed to insecticide spray at the following frequency:
 - Before spray
 - Second study to be done three days after insecticide exposure
 - Third study to be done after five days of insecticide exposure
- 2. Nerves to be studied (on the right side of the subjects):
 - Median (Motor)
 - Lateral popliteal (Motor)
 - Facial nerve
 - Median- Orthrodromic sensory
 - Sural- Antidromic sensory
 - Blink response-early Phase
- 3. Suggested machine for the study—MEDLEC MSA Machine
- 4. Proforma for clinical diagnosis:
Clinical Reg. No.....Date:.....
Name: Age: Sex:.....

Nerve conduction study

1. Right/Left MEDIAN (Motor): THENAR MUSCLES: SURF. ELE.

Wrist..... Elbow.....Supraclavicular.....
Amp.....
Latency..... msec..... msec..... msec.....m.v.
Distancycm cm cm
Conduction velocitymetres/sec. (Wrist to elbow)
Conduction velocitymetres/sec. (Elbow to supraclavicular region)

2. Right/Left ULLINar (Motor): Hypothemar muscles: Surf..... Ele.....

Wrist..... Elbow..... Supraclavicular.....Amp.....
Latency..... msec.....msec..... msec.....m.v.
Distancycm cm cm
Conduction velocity metres/sec (Wrist to elbow)
Conduction velocity metres/sec (Elbow to supraclavicular region)

3. Right/Left Lateral Popliteal: Ext. Dig. BR. : Surf. Ele.

Ankle..... Knee.....Amp.....
Latency..... msec.....msec..... msec.....m.v.
Distancecm cmcm
Conduction velocity metres/sec.

4. Right/Left Sural nerve (Antidormic-Sensory): Neelle Ele.

Amplitudeuv
Latency msec
Distancecm
Conduction velocity m/sec

5. Right/Left Median (Orthrodromic Sensory)

Stimulation- digital nerves-index finger
Recording at wrist: Needle Ele./Surf. Ele.
Amplitudeuv.
Latencymsec

6. Right/Left Ulnar (Orthrodromic Sensory)

Stimulation-digital nerves-index finger
Recording at wrist: Needle Ele./Surf. Ele.
Amplitude uv.
Latency msec

7. Right/Left Facial nerve

| Muscles | Latency..... | Amplitude..... | Distance..... |
|----------------|--------------|----------------|---------------|
| Orb. oris |msec | mv/uv | cm |
| Frontalis..... | msec | mv/uv | cm |
| Orb. oculi |msec | mv/uv | cm |

8. Needle Electromyography

- (i). Fibrillations..... FasciculationsInsertional activity
MystonicInterference patternAmplitude of motor units
- (ii). Fibrillations Fasciculations Insertional activity.....
MystonicInterference patternAmplitude of motor units
- (iii). Fibrillations FasciculationsInsertional activity.....
MystonicInterference pattern.....Amplitude of motor units

9. Blink response study

Needle electrode (Conc.): Right Orb. Oculi.

| | |
|----------------------|-------|
| Stimulation..... | |
| Latency..... | |
| Early Response | msec. |
| Late Response | msec. |

Signature of Medical Practitioner

Date :

(Seal)

Place :

Annexure 5

Distribution of nets (ITNs and LLINs) to householders and their consent

Project Title:.....

Name of the Institute and Address:

Names of the responsible Investigators:.....

I understand that my family and I have been asked to take part in the trial of a new insecticide in our village. I have been told that this study is being done to control mosquitoes/sand-flies and that my house should be provided with insecticide treated nets and my family should be asked to sleep inside the net. The period of study is months.

I am informed that the insecticide treated net causes no considerable risk or discomfort to human beings, if used at the recommended dose. I and all the adult members of the family are given necessary instructions about the safe use for self and children and proper storage of these nets.

I am told that my house/room might be modified to fit mosquito traps at the cost of the Investigators, but would be restored at the end of the study. I have been informed not to wash the net (s) supplied to us. During the study period I understand that the teams (Name of the Investigating Institute) may also visit our house even at odd hours for collection of mosquitoes and doing other tests on the nets.

I also understand that the Principal Investigator of the study can exclude me or my house from the study without my consent. However, I am also free to withdraw from the study without assigning any reason and without any cost implications thereof.

I have gone through the contents in the consent form, understood and agree to abide by them. I shall also apprise the members in my family of the contents in consent form and assure needed cooperation and precautions for the completion of the trial.

If I have any question about the study, I should contact (Name of the principal investigator) or (Name of the Investigator) at (Address of the Office/Institute) or for immediate medical help to the Medical Officer In charge (Name of the Medical Officer) , Primary Health Centre...../CHC..... in need.

I and members of my family agree to participate in the study voluntarily and not under duress or pressure or for remuneration.

Householder's signature/thumb impression Date:.....

House No.:.....Village:.....PHC/CHC:.....District:.....State:.....

Signature of Principal Investigator/Investigator

Place:

Date:

(This format should be translated into respective local language(s) in the study area and provided to the householder and read to him. A copy of the signed consent form should be given to the householder).

Annexure 6

Assessment of community perceptions on adverse effects and collateral benefits of insecticide treated nets (ITN) and long-lasting insecticide-treated nets (LLIN)

Date of supply..... and interview/discussion

1. Name of respondent: (Optional)
2. Age
3. Sex
4. Education status
5. Village name
6. Do you know why mosquito nets are used?
7. Do you use nets for protection for yourself/members of the family?
8. What are the other methods you use for protection?
9. Do you use any indigenous method for protection?
10. Are you aware whether something was provided for personal protection in your house?
If yes, when and why?
11. Generally how many people sleep inside the net(s)?
12. Do you sleep inside the net?
13. How does it smell?
14. Do you feel any of the following?
Skin irritation Nausea Vomiting Itching Headache
Drowsiness Eye irritation Difficulty in breathing Any other
15. Do the sleepers complain about suffocation?
16. Any fear of poisoning:
17. Observations/perceptions of the effect of insecticide-treated bed net or LLIN
 - on mosquito bites
 - on bed bugs
 - on head lice
 - on body lice
 - on domestic animals
 - Any other
18. Do you recommend use of the new insecticide-treated net in future? Yes/No
Reasons

Signature of Interviewer

Place:

Date:

(This format should be translated into respective local language/s in the study area and provided to the householder and read to him. A copy of the signed consent form should be given to the Householder).

Annexure 7

Form for tunnel bio-assay

Name of person doing bioassays:

Date of test: LN Code: Temp:..... °C RH: %

Test mosquito species and strain:

Age of mosquitoes:days

Test start time (hour/min): End time:

| | | Blood-fed females | | Unfed females | | Total | |
|--------------------------------|----------------------------|-------------------|------|---------------|------|-------|------|
| | | Alive | Dead | Alive | Dead | Alive | Dead |
| Control | Compartment 1 ^a | | | | | | |
| | Compartment 2 ^b | | | | | | |
| | Total | | | | | | |
| Treatment (LN) ^c | Compartment 1 | | | | | | |
| | Compartment 2 | | | | | | |
| | Total | | | | | | |

^aCompartment 1 refers to the long section of the tunnel where mosquitoes are released.

^bCompartment 2 refers to the section between the test netting and the animal bait

^cAdditional treatment rows to be added to record data when more than one sub-sample of the same net or netting samples from other nets are run in parallel.

Annexure 8

Questionnaire for community acceptability, physical integrity and washing methods of nets

Title of the project:

Name of Principle Investigator:

Name of Organization:

Name of Sponsor:

Five digit survey code (first two digits country; one digit village; two digits for sample: -----

Country State: District

Village Nearest town

Date of survey (DD/MM/YY):/...../.....

1. Net usage and acceptance

Information on net usage provided by:

- 1) User of this net
- 2) Caretaker of those using the net
- 3) Head of household
- 4) Other (specify)

Information on net usage:

- 1) Year-round and every night.
- 2) Year-round but occasionally.
- 3) Seasonally but every night.
- 4) Seasonally and occasionally

How is the net used?

- 1) Hanging over the bed
- 2) Hanging over sleeping mat/mattress on the ground
- 3) Other (specify)

Does sleeping under the net have any adverse or beneficial effect on you?

- 1) Yes
- 2) No

If Yes, describe the effect.

When was the last time you washed the net? (month)

How frequently you wash the net? (month)

How many times have you washed the net?

How was the net last washed?

Water:

1) cold2) warm 3) hot

With or without soap.....

Soap:

- 1) Village (local)-made soap
- 2) Commercial bar
- 3) Commercial powder
- 4) Mix of soap and powder

Rubbing against rocks/stone:

1) Yes 2) No

Where was the net dried after washing?

1) Inside 2) Outside under shade 3) Outside under the sun

2. Physical inspection of nets

2.1 Does net have holes?

1) Yes ... 2) No ...

If yes, use the following code for sizes of holes

- 1) hole smaller than will allow a thumb to pass through
- 2) a larger hole, but will not allow a closed fist to pass through
- 3) hole bigger than a closed fist

Total number of holes per net:

..... size 1
..... size 2
..... size 3

Total number of holes on:

..... lower half of the net
..... upper half of the net
..... roof

Total number of open/failed seams using the size coding provided above:

..... total size 1

..... total size 2

..... total size 3

Total number of repairs:

..... with stitches

..... with knots

..... with patches

Total number of holes due to burns? #

Aspect of net:

1) clean

2) a bit dirty

3) dirty

4) very dirty

3. Assessment of attrition rate

1. Number of nets of each size provided to the household in the beginning:

2. Number of nets physically present on the day of visit:

3. If a net is found lost to follow up, give main reason for loss of each net (s):

(Ask openly what happened to the nets and depending on the answer probe for other possibilities, e.g. lost, sold, given to relation or friend, worn out, burnt, and eaten by rats)

Record the number of nets remaining in the house and for each one record the number/size of holes and tears to give an indication of the rate of wear and tear⁶

Name of investigator

Signature

⁶This is a check on the truthfulness for the reasons given for the loss. We need to distinguish loss due to wear and tear (true attrition) from loss due to misdemeanour (e.g. selling on). If the remaining nets are quite holed, loss due to wear and tear would appear genuine.

Annexure 9

Assessment of adverse effects, if any, among impregnators of nets

Title of the project:

Type of treatment:

1. Code # of the respondent impregnator:

2. Age of the respondent (years):

3. Education status:

4. Date(s) of net impregnation (dd/mm/yy):

5. No. of nets treated:

6. Date(s) of side-effects survey (dd/mm/yy):

7. Following treatment of nets did you observe any of the following effects (record past and present experience)

| | | Yes | No | If yes, duration |
|----|--|-----|----|------------------|
| a | Itching of your skin | | | |
| b | Facial burning/tingling | | | |
| c | numbness or a loss of physical sensation and/or tingling of your skin (paraesthesia) | | | |
| d | Sneezing | | | |
| e | Liquid discharge from your nose | | | |
| f | Feeling of headache | | | |
| g | Symptom of nausea | | | |
| h | Eye irritation | | | |
| i | Tears coming from your eyes | | | |
| j. | Experience bad smell during use of nets | | | |
| k. | Body rashes | | | |

Signature of interviewer

Date (dd/mm/yy):

Annexure 10

Assessment of adverse effects, if any, among In/treated net users

Title of the project:

1. Household Code #:

Net Code:

2. Date(s) of receipt of ITN/LLIN (dd/mm/yy):

3. Number of nets provided by project:

4. Number of coded-net users in your house:

3. Following the use of nets did you observe any of the following effects (record past and present experience)

| | | Yes | No | If yes, duration |
|----|--|-----|----|------------------|
| a | Itching of your skin | | | |
| b | Facial burning/tingling | | | |
| c | numbness or a loss of physical sensation and/or tingling of your skin (paraesthesia) | | | |
| d | Sneezing | | | |
| e | Liquid discharge from your nose | | | |
| f | Feeling of headache | | | |
| g | Symptom of nausea | | | |
| h | Eye irritation | | | |
| i | Tears coming from your eyes | | | |
| j. | Experience bad smell during use of nets | | | |
| k. | Body rashes | | | |

l. Any other symptoms, please specify?

m. Your overall experience and whether you will use your net regularly?

n. If the respondent answers positive to any of the questions mentioned above, ask if he/she had reported to a physician for medical attention

Signature of interviewer

Date (dd/mm/yy):

5. APPENDIX

Calculation of Doses

1. Measurement of sprayable surface area of a room

Formula = $(L \times W + W \times H + H \times L) \times 2 - W \times L$

Example: L: Length of wall = 12' W
Width of wall = 10'
Height = 8'

Area = $(12 \times 10 + 10 \times 8 + 8 \times 12) \times 2 - 10 \times 12$
 $(120 + 80 + 96) \times 2 - 120 = 296 \times 2 - 120$
 $592 - 120$
 $472 \text{ ft}^2 = 0.472 \text{ m}^2$

Note: For measuring artificial surfaces only length and width should be calculated.

2. Requirement for the preparation of spray suspension from wettable powders

Amount of wettable powders (WP) or water-dispersible powder (WDP) required for the preparation of approximately 10 litres of spray suspension.

The general formula followed

$$X = A \times B \times D / C$$

X = amount of water-dispersible powder required

A = percentage concentration desired

B = quantity of spray desired

C = percentage concentration of water-dispersible powder

D = 1 (when X and B are expressed in kg and litres)

3. Requirement for the preparation of spray suspension from dust

The general formula followed

$$X = \frac{A \times 100}{B}$$

X = amount of dust required

A = dosage (kg/ha)

B = percentage concentration of dust

4. Measurement of surface area of mosquito breeding waters

(a) Rectangular/square area

Formula = $L \times W = \text{Surface area}$

Example = $4.5 \text{ m} \times 3 \text{ m} = 13.5 \text{ m}^2$

Volume of water = Surface area x Depth*

(*The dose of Temephos or Fenthion may be doubled or tripled in case water bodies having more than 50 cm depth)

(b) Measurement of round surface area

Formula = πr^2 or $22/7 \times r \times r$

e.g. Diameter of well/pit = 3 m

Radius of well/pit = 1.5 m

Area = $22/7 \times 1.5 \text{ m} \times 1.5 \text{ m}$
= $3.1 \times 1.5 \times 1.5 = 6.97 \text{ m}^2$

(c) Measurement of volume of water in circular pit/well

Formula = $\pi r^2 \times \text{depth}$

e.g. Diameter of well/pit = 3 m

Radius of well/pit = 1.5 m

Depth of well/pit = 0.30 m

$\pi r^2 \times \text{depth}$ = $3.1 \times 1.5 \times 1.5 \times 0.30 \text{ m}$
= 2.092 m²

Volume of water = 2.092 litres of water

or

$\pi r^2 \times \text{depth}$ = $3.1 \times 150 \text{ cm.} \times 150 \text{ cm} \times 30 \text{ cm}$
= 2092500 cm²

Volume of water = 2092500/1000

= 2092 litres of water

(d) Measurement in number of hectares in areas of different linear dimensions

$$\text{Length (m) x Width (m)}$$

$$\text{Area (hectares)} = \frac{\text{Length (m) x Width (m)}}{10000}$$

e.g., Length of breeding water = 1600 m

Width of breeding water = 25 m

$$\text{Area} = \frac{1600 \times 25}{10000} = \frac{40000}{10000} = 4 \text{ hectares}$$

or

$$\text{Length (ft) x Width (ft)}$$

$$\text{Area (acres)} = \frac{\text{Length (ft) x Width (ft)}}{43560}$$

e.g., Length of breeding water = 3600 ft

Width of breeding water = 500 ft

$$\text{Area} = \frac{3600 \times 500}{43560} = \frac{1800000}{43560} = 41.3 \text{ acres}$$

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Acknowledgement

The ICMR and the NVBDCP acknowledge with thanks the cooperation and contributions of the Central Insecticide Board & Registration Committee, Faridabad and National communicable Disease Centre, Delhi. The sincere efforts of the Expert Group and the Sub-Committee in drafting the revised Common Protocol are highly appreciated with special gratitude to Prof. M. K. K. Pillai, Chairman, the Expert Group. The ICMR and the NVBDCP express their appreciation to Dr. M. Kalyanasundaram, Scientist G, Dr. S. L. Hoti, Scientist F, Dr. G. Rajendran, Scientist D and Dr. R. Srinivasan, Scientist D, Vector Control Research Centre, Puducherry for their help in updating the document.



Dipper sampling of larval population in a breeding habitat