सत्यमेव जयते

Common Protocol for Uniform Evaluation of Public Health Pesticides for use in Vector Control

Third Edition

Indian Council of Medical Research
(Department of Health Research)

National Centre for Vector Borne Diseases Control

National Centre for Disease Control

Central Insecticides Board & Registration Committee

2023

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The third edition of "Common Protocol for Uniform Evaluation of Public Health Pesticides for use in Vector Control" has been developed in consultation with stakeholders namely the Indian Council of Medical Research (ICMR), National Centre for Vector Borne Diseases Control (NCVBDC), National Centre for Disease Control (NCDC) and Central Insecticides Board & Registration Committee (CIBRC) and subject experts. The ICMR, the NCVBDC, the NCDC and CIBRC acknowledge the sincere efforts and contributions of the Drafting Committee and its members in formulating the third edition of the Common Protocol. Sincere gratitude to Late Dr. Shiv Lal, former Special DGHS and Director NCVBDC who extended his unflinching support and guidance to this exercise. Special thanks are due to Dr. P. Jambulingam, Former Director, ICMR-Vector Control Research Centre, for chairing the committee meetings and for his critical review of all chapters and to Dr. K. Gunasekaran, Scientist G (Retd.), and Dr. C. Sadanandane, Scientist-D (Retd.), ICMR-VCRC, Dr. K. Raghavendra, Scientist G (Retd.), ICMR-NIMR, for their dedication and inputs for revising the chapters of the protocol.The support from the scientific staff of ICMR-NIMR and ICMR-VCRC is greatly appreciated.

The coordination and committed support by Dr. Manju Rahi, Director, ICMR-VCRC and Programme Officer (VBDs), ECD, ICMR in initiating this exercise and for successfully culminating the efforts to bring out the third edition of the Common Protocol are appreciated.

Preface

Vector control is a proven strategy for reducing transmission and controlling arthropod-borne diseases. Insecticides have proven to be an effective tool in vector control as these have significantly contributed to control and elimination of a few major vector-borne diseases in different regions worldwide. Insecticides will continue to have a prominent and relevant role in national vector control programmes aimed at curbing vector-borne diseases by limiting their transmission.

To cater to changing ecological situations, continuously altering disease paradigms and emerging insecticide resistance in vector species, there is a need for new insecticides/insecticide formulations which show adequate biological efficacy and human and environmental safety. For making informed selections of new insecticides to be used in different strategies for public health under the national programmes, to keep up with technological advancements in vector control tools, and to fill gaps in implementation, an updated common protocol is required for insecticide evaluation. Therefore, the Indian Council of Medical Research (ICMR) and the National Centre for Vector Borne Diseases Control (NCVBDC) had taken the onus to evaluate and introduce new insecticides or formulations and insecticide-treated/incorporated materials into the national vector-borne disease control programme. The new products are based on robust scientific evaluations via laboratory and field trials on their bio-efficacy and effectiveness (on target and non-target organisms).

Prof. Balram Bhargava, Former Director General, ICMR, is credited for successfully initiating the development of the revised protocol and for constant encouragement. With tremendous support from Dr. Rajiv Bahl, the current Director General, ICMR, the Drafting Committee has developed the common protocol in its revised and updated third edition under the chairmanship of Dr. P. Jambulingam, Former Director, ICMR-Vector Control and Research Centre (ICMR-VCRC). The committee elicited contributions from scientists of NCVBDC, National Centre for Disease Control (NCDC), Central Insecticide Board & Registration Committee (CIBRC), ICMR and its institutes. This document was technically examined for ease of implementation by Dr. K. Gunasekaran, Dr. K. Raghavendra, Dr. C. Sadanandane, Late Dr. Ram Singh, Dr. Sonai T. Rajan, Mr. Subhash Chand, Dr. Tenzin Dikid and Dr. Kalpana Baruah.

To conclude, I take this opportunity to express gratitude to all the members of the Drafting Committee, scientists, and other contributors for their dedicated efforts for successful completion of this document.

Dr. Manju Rahi
Director
ICMR-Vector Control Research Centre
and
Programme Officer (VBDs)
Division of Epidemiology and Communicable Diseases
Indian Council of Medical Research

सत्यमेव जयते

डॉ. राजीव बहल, एमडी, पीएचडी
DR. RAJIV BAHL, MD, PhD

INDIAN COUNCIL OF MEDICAL RESEARCH

सचिव, भारत सरकार
स्वास्थ्य अनुसंधान विभाग
स्वास्थ्य एवं परिवार कल्याण मंत्रालय एवं
महानिदेशक
भारतीय आयुर्विज्ञान अनुसंधान परिषद

Secretary, Government of India
Department of Health Research
Ministry of Health & Family Welfare &
Director-General
Indian Council of Medical Research

FOREWORD

Vector control remains a cornerstone strategy in reducing disease transmission and managing arthropod-borne diseases. Insecticides, in particular, have been instrumental in effectively combating major vector-borne diseases in various regions across the globe. They continue to play a pivotal role in our nationwide vector control programs.

The Indian Council of Medical Research (ICMR) and National Centre for Vector Borne Diseases Control (NCVBDC) have played a significant role in evaluating various insecticides as indoor sprays, larvicides and insecticidal nets, bacterial pesticides and growth regulators with a focus on continuously developing newer effective tools for vector control in public health.

In light of changing ecological dynamics, shifting disease paradigms, and the emergence of insecticide resistance in vector species, the demand for novel insecticides and formulations that are both biologically effective and environmentally friendly has become apparent. To facilitate informed choices of these novel insecticides, and to keep pace with technological advances in vector control tools an updated common protocol for insecticide evaluation is a pressing need.

In recognition of this need, ICMR in collaboration with NCVBDC, National Centre for Disease Control (NCDC) and Central Insecticides Board & Registration Committee (CIB&RC) has updated the Common protocol (second revised version) for evaluating existing and newer vector control interventions and successfully brought out the *3rd Edition of the Common Protocol for uniform evaluation of public health pesticides for use in vector control.* Drawing from the knowledge and experience acquired through previous editions, it incorporates the latest scientific advancements to meet the evolving challenges posed by vector-borne diseases and pesticide resistance.

The new products will be subjected to rigorous scientific assessments through laboratory and field trials. The outcomes of these robust evaluations will significantly assist NCVBDC, nodal state agencies, and other stakeholders in choosing the most suitable insecticides for vector-borne disease control. Additionally, these new insecticides will undergo registration with the (CIB&RC) for use in public health.

I am confident that this updated third edition of uniform evaluation methodology via multicentric laboratory and field trials will facilitate NCVBDC and other stakeholders to robustly select the insecticide for control of vector-borne diseases with the aim of disease elimination.

I congratulate the participating organizations and the Drafting committee for appraising the Common protocol for use in evaluation of public health pesticides products.

Rajiv Bahl

(Dr. Rajiv Bahl)

V. Ramalingaswamy Bhawan, Ansari Nagar, New Delhi-110029
Tel. : 011-26588204, Website : www.icmr.gov.in, E-mail : secy-dg@icmr.gov.in

भारत सरकार
Government of India
कृषि एवं किसान कल्याण मंत्रालय
Ministry of Agriculture & Farmers Welfare
कृषि, सहकारिता एवं किसान कल्याण विभाग
Department of Agriculture, Co-operation & Farmers Welfare
वनस्पति संरक्षण, संगरोध एवं संग्रह निदेशालय
DIRECTORATE OF PLANT PROTECTION, QUARANTINE & STORAGE
केंद्रीय कीटनाशी बोर्ड एवम पंजीकरण समिति
Central Insecticides Board and Registration Committee
एन. एच. 4, फरीदाबाद (हरियाणा)-121001
N.H. IV, FARIDABAD (HARYANA)-121001

MESSAGE

I am pleased that the Indian Council of Medical Research (ICMR), National Centre for Vector Borne Diseases Control (NCVBDC) and National Centre for Disease Control (NCDC) has developed the third edition of "Common Protocol for Uniform Evaluation of Public Health Pesticides for use in Vector Control". The common protocol shall evaluate new pesticides formulations as a public health measure for vector control to manage vector-borne diseases. We are pleased to be a part of this vision since the new insecticides or insecticide formulations found promising in field trials shall be registered with the Central Insecticide Board &Registration Committee (CIB&RC), Govt. of India for regulated use in public health in vector control programmes.

I congratulate ICMR, NCVBDC and NCDC for this initiative to address implementation of structured evaluation methods to assess efficacy for use in vector control. Robust regulation of new pesticides shall play a crucial role in improving vector control and management and propelling success in the right direction with focus on disease elimination in public health.

Dr Sanjay Arya, Secretary,
Central Insecticide Board & Registration Committee

प्रो.(डॉ.) अतुल गोयल
Prof. (Dr.) ATUL GOEL
MD (Med.)
स्वास्थ्य सेवा महानिदेशक
DIRECTOR GENERAL OF HEALTH SERVICES

भारत सरकार
स्वास्थ्य एवं परिवार कल्याण मंत्रालय
स्वास्थ्य सेवा महानिदेशालय
Government of India
Ministry of Health & Family Welfare
Directorate General of Health Services

MESSAGE

Vector Borne Diseases (VBDs) continue to have a significant impact on the health of people in India thereby affecting their socio-economic well-being. Although, significant advancements have been made in the field of diagnosis and treatment of VBDs, vector control remains the key component in prevention and control of VBDs. To overcome challenges related to efficacy and human safety of public health pesticides in various geographical regions of the Country, newer insecticides are included under the National program following Standard Operating Procedures.

It is immensely satisfying to note that Indian Council of Medical Research (ICMR) in collaboration with NCVBDC, National Centre for Disease Control (NCDC) and Central Insecticides Board & Registration Committee (CIB&RC) has updated the existing Revised Common Protocol (2014) and developed this 3rd Edition of 'Common Protocol for Uniform Evaluation of Public Health Pesticides for use in Vector Control'. This document elicits the common methodology for the evaluation of pesticides for vector control to manage VBDs. This edition has been developed in consultation with Entomologists, stakeholders and Experts from reputed National organizations.

I congratulate all experts from ICMR, NCVBDC, NCDC and CIB&RC for their significant contribution towards development of the 3rd edition of 'Common Protocol for Uniform Evaluation of Public Health Pesticides for use in Vector Control'. I hope that this document will facilitate the program managers and other stakeholders for introduction and use of newer public health pesticides for prevention and control of vector-borne diseases and help in achieving disease specific targets.

(Atul Goel)

Room No. 446-A, Nirman Bhawan, New Delhi-110108
Tel. No.: 011-23061063, 23061438, Fax No.: 011-23061924, email: dghs@nic.in

Balram Bhargava
Professor & Head, Chief CTC
MD, DM, FRCP (Glasg.), FRCP (Edin.),
FACC, FAHA, FAMS, FNASc

Department of Cardiology
Cardiothoracic Sciences Centre
All India Institute of Medical Sciences
Ansari Nagar, New Delhi-110 029 (INDIA).
Tel.: 011-2659 3322, 2659 4890
Secretary, 9868398175
Fax: 91-11-26588641, 26588663
E-mail: balrambhargava@yahoo.com

29th September, 2023

MESSAGE

Vector control is central to the success of vector-borne disease control programmes aiming at vector-borne disease elimination. To address multitude of changes in disease paradigms and ecology together with threat of insecticide resistance in insect vectors, newer effective vector control tools are urgently needed. On this note, the Indian Council of Medical Research (ICMR), National Centre for Vector Borne Diseases Control (NCVBDC), National Centre for Disease Control (NCDC) and Central Insecticide Board & Registration Committee (CIB & RC) initiated the development of a uniform common protocol for evaluating newer vector control interventions with a vision to fast-track disease elimination programmes.

I am delighted that this updated third edition of the uniform evaluation methodology via multicentric laboratory and field trials has come to a fruitful completion. The revised and updated document will facilitate NCVBDC and other stakeholders to select the insecticide for control of vector borne diseases. I congratulate committee members that collaborated to update the document using their invaluable expertise.

Balram Bhargava

Padmashri Prof. (Dr.) Balram Bhargava
Chief of Cardiothoracic Centre, AIIMS
All India Institute of Medical Sciences, New Delhi
Former, Secretary Department of Health Research &
Director General, Indian Council of Medical Research

डॉ. तनु जैन
निदेशक
DR. TANU JAIN
Director
MBBS, M.D. (Community Medicine)
Tel.: 011-23921086
E-mail: dir.ncvbdc@gmail.com
tanu.jain69@nic.in

सत्यमेव जयते

राष्ट्रीय वैक्टर जनित रोग नियंत्रण केंद्र
(स्वास्थ्य सेवा महानिदेशालय)
स्वास्थ्य एवं परिवार कल्याण मन्त्रालय, भारत सरकार
NATIONAL CENTER FOR VECTOR BORNE DISEASES CONTROL
(Directorate General of Health Services)
Ministry of Health & Family Welfare, Govt. of India

MESSAGE

The National Center for Vector Borne Diseases Control (NCVBDC) under Ministry of Health and Family Welfare, GoI deals with prevention and control of VBDs namely Malaria, Dengue, Chikungunya, Kala-azar, Lymphatic filariasis and Japanese encephalitis in the Country. Vector control is an integral part of NCVBDC strategies since its inception. For success of programme implementation, public health pesticides are indispensable tools for vector control. Thus, it becomes imperative to evaluate newer effective vector control and management tools in view of development of resistance in vectors towards the insecticides under use.

In view of changing ecological dynamics, disease paradigms, newer diseases and emergence of insecticide resistance in vectors, there is a need for biologically effective and environmentally friendly novel insecticides and formulations. To overcome these challenges, both NCVBDC and Indian Council of Medical Research (ICMR) played a significant collaborative role in evaluating newer insecticides, bio-larvicides and growth regulators for use under the National programme.

I am happy to know that ICMR, NCVBDC, National Centre for Disease Control (NCDC) and Central Insecticide Board & Registration Committee (CIB&RC) put their efforts in drafting of the 3rd edition of 'Common Protocol for Uniform Evaluation of Public Health Pesticides for use in Vector Control' by updating the existing Revised Common Protocol (2014). This edition of uniform evaluation methodology via multicentric laboratory and field trials will facilitate the stakeholders to evaluate insecticides for control of VBDs. I would like to congratulate the Committee members and Experts from various organizations for their contribution in developing this updated edition. I hope this will make the process of introduction of newer insecticides easier and more effective.

(Dr. Tanu Jain)

Contents

Abbreviations

AI	Active Ingredient
ANOVA	Analysis of Variance
API	Annual Parasite Incidence
ASB	Attractive Sugar Baits
ATSB	Attractive Toxic Sugar Baits
CDC	Center for Disease Control
CFV	Control Flow Valve
CHC	Community Health Centre
CI	Confidence Interval
CIBRC	Central Insecticide Board & Registration Committee
CIPAC	Collaborative International Pesticides Analytical Council
CRT	Cluster Randomised Trial
CPT	Complete Protection Time
CS	Capsule Suspension
DC	Diagnostic or Discriminating Concentration
ECoP	Environmental Codes of Practice
ED	Effective Dosage
EDPT	Early Diagnosis and Prompt Treatment
EIR	Entomological Inoculation Rate
ELISA	Enzyme-Linked Immunosorbent Assay
FT_{50}	First Takeoff (median time in seconds)
GLM	Generalized Linear Model
GLP	Good Laboratory Practice
HBI	Human Blood Index
HDPE	High-Density Polyethylene
HLC	Human Landing Catches
ICMR	Indian Council of Medical Research
IE	Inhibition of adult Emergence
IGR	Insect Growth Regulator
IRS	Indoor Residual Spraying
ITN	Insecticide Treated Net
IVM	Integrated Vector Management
KD	Knock Down
LD_{50}	Median Lethal Dose

LLIN/LN	Long Lasting Insecticidal Net
LSD	Least Significant Difference
MAP	Malaria Action Plan
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
NCVBDC	National Centre for Vector Borne Diseases Control
PBO	Piperonyl Butoxide
PCR	Polymerase Chain Reaction
PHC	Primary Health Centre
PHPs	Public Health Pesticides
PMHD	Per Man-Hour Density
PPE	Personal Protective Equipment
PPM	Parts Per Million
PPS	Probability Proportional to Size
PVC	Polyvinyl Chloride
RDT	Rapid Diagnostic Test
RH	Relative Humidity
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
WP	Wettable Powder
WDP	Water Dispersible Powder
WP-SB	Wettable Powder in Soluble Bags
WHO	World Health Organization
WHO PQT/VCP	WHO Pre-Qualification Unit/Vector Control Products
WHOPES	WHO Pesticide Evaluation Scheme

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. The National Center for Vector Borne Diseases Control (NCVBDC), formerly known as the National Vector Borne Diseases Control Programme (NVBDCP), the nodal agency of the Government of India for the control of vector-borne diseases, has endorsed the strategy. Among the available vector control methods, chemical control remains 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), use of long-lasting insecticidal nets (LLINs) and a list of household insecticide formulations for personal protection. In India, the NCVBDC largely relies on site-specific chemical control, using insecticides of different classes. A major impediment to this method has been the development of resistance in vector species to the insecticides in use, which necessitates replacement of the insecticides with new insecticides showing adequate biological efficacy and human and environmental safety. The NCVBDC has the responsibility to introduce new insecticides or insecticide formulations and insecticide treated/ incorporated materials to the vector-borne diseases control programme based on the results of scientific evaluation of the products. To meet the continued demand, National Centre for Disease Control (NCDC) and institutes of the Indian Council of Medical Research (ICMR) will carry out laboratory and field trials to evaluate new insecticide compounds or formulations for their bio-efficacy and effectiveness on target and safety of non-target organisms. Field testing of such products needs to be carried out in multi-centric mode at different sites of variable eco-epidemiology (geographical locations) to ascertain their suitability for use in diverse situations/conditions in the country. It is mandatory that the new insecticides or insecticide formulations found promising in field trials are registered with the Central Insecticide Board & Registration Committee (CIBRC), Directorate of Plant Protection, Quarantine and Storage (DPPQS), Ministry of Agriculture and Farmers Welfare, Govt. of India for use in public health for NCVBDC to consider their use in vector control programmes. The WHO Pesticide Evaluation Scheme (WHOPES) was the only international programme engaged in promoting and evaluating pesticides for public health use by providing technical assistance to the member countries and encouraging the industries to develop alternate insecticides for vector control programme. Now WHOPES has been replaced by WHO Pre-qualification Unit/ Vector Control Products (WHO PQT/VCP).

1.1. Need for a common protocol for uniform evaluation

As stated above, NCVBDC has been the nodal agency for introduction of new public health pesticide products (PHPs), which include insecticides / insecticide formulations / LLINs / bio-larvicides registered with the CIBRC, for vector control under the national programme based on their need, suitability and adaptability to Indian conditions assessed through laboratory and multi-centric

field trials by recognised universities and research organizations (ICMR/ NCDC). For the products to meet the mandatory requirement of CIBRC's registration and to be approved by NCVBDC for use in the national vector control programme, it is necessary to generate entomological data (i.e., bio-efficacy data) and data on the impact on disease incidence / prevalence (https://ppqs.gov.in/divisions/central-insecticides-board-registration-committee). The WHO PQT/VCP-listed pesticides are approved by the NCVBDC only after large-scale and multi-centric field testing/evaluation in India and registration with the CIBRC.

Although there are general guidelines by the erstwhile WHOPES for the evaluation of insecticides, considering diverse eco-epidemiological conditions / situations prevailing in India and the varying capacity of the institutes involved in insecticide / product evaluation in terms of expertise, it is important to harmonize the methodologies for evaluation of vector control products. This uniformity will facilitate the comparison of results generated by different institutes and decisions on the suitability of products for use in Indian conditions. Therefore, the development of a common protocol for uniform evaluation of public health pesticides, including bio-larvicides, becomes imperative. Keeping this in view and in line with the WHOPES guidelines for insecticide evaluation, a common protocol was jointly prepared by the ICMR-National Institute for Malaria Research (ICMR-NIMR), New Delhi, and the ICMR-Vector Control Research Centre (ICMR-VCRC), Puducherry in the year 2005 (First edition), which was revised / updated in 2014 (Second edition) by a Sub-Committee constituted by the Director General, ICMR.

The first and the second edition of the protocol were designed based on the information/ knowledge on insecticide classes, insecticide formulations, mode of action, long-lasting insecticidal net (LLIN) technology, evaluation parameters, and testing procedures available at that time. Given the subsequent developments, particularly of widespread development of resistance to insecticides, including pyrethroids among disease vectors, innovation of new products with novel mode of action, including slow-acting insecticides and chemicals that affect mosquito blood feeding or reproductive output, recommendations for improved trial designs and additional assays for detection of insecticide resistance etc., it was decided in the ICMR Expert group meeting to further revise/update the common protocol. Thus, the current (Third) edition of the common protocol is an expanded and updated version of the earlier editions of the protocol.

The protocol is prepared for testing and evaluation of insecticides or insecticide formulations against mosquito vectors. However, the basic principles outlined in the protocol may be the same for the evaluation of insecticides and insecticide formulations against other disease vectors.

It is envisioned that the protocol would form a basis that may be used for the registration of newer vector control products by the country registration authorities.

1.2. Insecticides Evaluation Phases

The evaluation of insecticides is performed in three Phases.

Phase I (Laboratory evaluation)

The efficacy of new technical PHPs or their formulations is evaluated under controlled conditions in the laboratory using insectary-reared characterized vector mosquitoes. This phase includes studies on efficacy and persistence, diagnostic concentration, and cross-resistance in vectors.

- Phase I evaluation may not be necessary for WHO-pre-qualified PHPs
- Sponsoring industries/agencies (national/international) must provide data on human/ mammalian toxicity and environmental safety and risk assessment documents.

Phase II (Small-scale field evaluation)

Small-scale evaluation is carried out in the field or simulated field conditions/experimental huts to determine the field dosage and frequency of application of the product tested. This phase provides information on efficacy in field conditions, including safety to operators and inhabitants.

It is also an opportunity to verify the effect on relevant non-target organisms, as given below (Box 1.1). 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 WHO-PQ, need not be repeated in India.
- PHPs to be tested must have ethical clearance from the respective institutional ethics committees.
- Informed consent should be obtained from the human volunteers associated with the evaluation.

Phase III (Large-scale field evaluation)

In this phase, the product is evaluated in the field at the dosage recommended in Phase II on a large-scale [village(s)-scale] against disease vectors prevalent in the area. This phase assesses the impact on entomological parameters, disease incidence/prevalence, relevant target and non-target organisms, community acceptance and operational safety.

- Phase III evaluation should be carried out at least in three eco-epidemiological settings (multi-centric).
- The trial should have ethical clearance from their respective institutional ethics committees'.
- Informed consent should be obtained from the spraymen/households associated with the evaluation.

Box 1.1: List of commonly available non-target organisms (NTOs) against which the effect of the insecticides may be evaluated.

In order to maintain a uniformity in the 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)

Others:

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

1.3. Product Safety and Specifications

Prior to the evaluation of any product at different phases (Phase I, Phase II and Phase III), data on safety and specifications of the technical grade material or the related formulations generated/ provided by WHO/a Government accredited/recognized/certified laboratory and the Generic risk assessment – Human Health of the products generated by WHO should be provided by the manufacturer/ sponsor of the product (Available at: https://main.icmr.nic.in/sites/default/files/upload_ documents/Checklist_for_evaluation_of_PHP.pdf).

NCVBDC guidelines (Available at: https://nvbdcp.gov.in/WriteReadData/l892s/Guidelines-for-ITNS-LLINS.pdf) be referred to for storage, safe handling, transportation, distribution of ITNs/LLINs, and the WHO document (Available at: https://apps.who.int/iris/bitstream/handle/10665/310862/9789241550499-eng.pdf) for safe disposal of ITNs/LLINs.

2

Indoor Residual Spraying (IRS)

Indoor residual spraying (IRS) is one of the effective vector control options. In India, it is extensively used to control malaria and kala-azar transmission. Indoor resting (endophilic) mosquitoes effortlessly pick up the lethal dosage of insecticide through their tarsal contact. 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) and insecticide formulations in soluble bags, e.g., WP-SB, WG-SB, to the interior surfaces of houses and other resting shelters of vector (s). The effectiveness of IRS depends on the following conditions:

- Extent of endophily (mosquitoes which rest indoors before and/or after a blood meal) and endophagy (even an exophilic mosquito species, if endophagic, is likely to come in contact with sprayed surfaces, as fed mosquitoes tend to rest for a while after a blood meal).
- Application of adequate dosage of the insecticide formulation
- Adequate coverage of sprayable surfaces in the habitats such as walls, eaves, ceiling/ roof, and other potential resting places of the disease vectors
- Residual activity of the insecticide formulation throughout the transmission period

For the insecticides/insecticide formulations, which are evaluated for the first time (new insecticides/ formulations), specifications of the technical material of the compound should be provided by the sponsor of the product. The sponsor should also provide data on toxicological indices of safety for humans and non-target organisms, especially against domestic/ pet animals. In other words, the material safety data sheet {MSDS} of the new insecticide/ formulations should be provided.

Any new insecticide or formulation developed for IRS needs to pass through three phases of evaluation: Phase I (Laboratory evaluation), Phase II (Simulated field or small-scale field evaluation or evaluation in experimental huts), and Phase III (Large-scale field evaluation).

The new insecticides or formulations that show promising activity in laboratory evaluation (Phase I) will be considered for small-scale (Phase II) and subsequently large-scale (Phase III) field evaluation. The WHO pre-qualified insecticides/formulations can be directly taken up for Phase II evaluation.

2.1. Phase I - Laboratory evaluation

Duration: 6 months

Objectives

- To determine the intrinsic insecticidal activity of the active ingredient of a given insecticide against the target vector species by estimating LD_{50} and $LD_{99.}$
- To establish a dose-response relationship and the discriminating concentration of the active ingredient for monitoring resistance to the insecticide and cross-resistance to other classes of insecticides in field use.
- To assess irritant and excito-repellent properties of the insecticide by determining ('Time to first take off') FT_{50} and FT_{95} after exposure to the insecticide-treated substrates.
- To determine the efficacy and residual activity of the insecticide deposits on different substrates.

2.1.1. Intrinsic toxicity

Objective

- To determine the intrinsic insecticidal activity of the active ingredient of a given insecticide against the target vector species

The activity is tested by topical application of an active ingredient to isolate toxicity from confounding effects resulting from insect behaviour (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.
- Fifty non-blood-fed susceptible female mosquitoes are weighed initially to determine the average live-weight of a mosquito.
- A constant volume of 0.1 μl containing a known insecticide concentration is applied on the pronotum using a pipette. Adding larger volumes should be avoided as it may cause higher mortality due to solvent toxicity.
- For the treatment group, two batches of 25 mosquitoes each will be tested with each insecticide. Parallel control of two batches of 25 female mosquitoes, each treated with 0.1 μl of pure acetone, will be kept.
- The mosquitoes are anaesthetized using CO_2 for 30 seconds and placed on a plate under cooling at 4°C to maintain anesthesia during the manipulations.
- After testing with a wide range of concentrations ($\sim$10 concentrations that give a mortality range of 0 to 100%), a narrow range with $\sim$six concentrations (at least one concentration should kill 100% mosquitoes, two should kill $>$50%, one concentration should kill around 50%, and two concentrations should kill $<$50%) should be selected and used per test. A total of 50 susceptible, non-blood-fed, 3–5 days-old female mosquitoes are tested at each concentration.
- After dosing, the females are transferred into clean holding cups provided with cotton wool soaked in 10% sugar solution and held for 24 h at 27$\pm$2 °C temperature and 80 $\pm$ 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 solutions should be prepared and used.

- The dose is expressed in nanograms of active ingredient per mg of body weight of live mosquito.
- Log dose-probit regression (Finney, 1971) analyzes the relationship between dose and mortality. LD_{50} and LD_{90} and their 95% confidence limits are determined using appropriate statistical software. If mortality exceeds 20% in control replicates, the test is rejected. If mortality in the controls is between 5% and 20%, the treated mortality is corrected with the control mortality using the Abbott's formula (Abbott, 1925), as given below:

$$\%\ \text{Corrected mortality} = \frac{(\%\ \text{Treated mortality} - \%\ \text{Control mortality})}{(100 - \%\ \text{Control mortality})} \times 100$$

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

2.1.2. Discriminating (or diagnostic) concentration

The discriminating (or diagnostic) concentration (DC) is the one used for susceptibility tests to detect or monitor the presence of resistance in the target vector species to a given insecticide. WHO-recommended DC is available for majority of the insecticides in use (https://apps.who.int/iris/bitstream/handle/10665/352616/9789240045200-eng.pdf), but, for newer molecules, the DC needs to be determined through log dose-probit mortality response analysis.

Preparation of insecticide-impregnated papers

- Discriminating concentration is determined by exposing the target mosquito species to a graded series of concentrations of insecticide (technical grade) impregnated on Whatman® No. 1 filter-papers.
- According to the insecticide to be tested, appropriate carrier oil should be chosen to prepare a solvent solution (carrier oil + acetone): Risella® (Shell) oil for DDT, olive oil for organophosphates and carbamates, and silicon oil (e.g., BDH Dow Corning® 556) for pyrethroids.
- Degree of purity of the insecticide (technical grade) to be used should be noted as this is important to calculate the quantity of active ingredient to be mixed with the solvent.
- Concentration of insecticide necessary for impregnation should be chosen.
- Technical grade insecticide is dissolved in a non-volatile carrier oil, and 0.8 ml of this is mixed with 1.2 ml of acetone (2 ml is the standard volume required per paper), and applied to rectangular pieces of Whatman® No. 1 filter-paper measuring 12 × 15 cm.
- The carrier oil allows formation of a stable, thin, and homogeneous layer of the insecticide on the filter-paper and also prevents the crystallization of active ingredients.
- Since acetone is volatile, the insecticide concentration is normally expressed as % of active ingredient (ai) per unit volume of carrier oil on the filter-paper.
- Example: Papers are impregnated at 3.6 mg/cm² of the carrier oil, i.e., 648 mg/paper of 0.018 m² 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 grade insecticide or 367 mg/m² of concentration.
- The filter-paper on the rough side is impregnated by pipetting the insecticide solution evenly onto the paper pinned on cardboard. The smooth side is labelled with details of impregnation, such as the name of insecticide, concentration, date of impregnation, etc.
- The papers after impregnation are air dried for 24 h and used for testing. The impregnated paper should not be used more than six times (WHO, 2016).

Determination of discriminating (or diagnostic) concentration

Mosquitoes are exposed to graded series of the insecticide concentrations impregnated on filter-papers. (The detailed test procedure using WHO tube method (filter-paper assay) and WHO bottle

assay is given in Box 2.2 and Box 2.3, respectively). 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, one gives around 50% and at least two give between 5% and 50% mortality.

The concentration/ mortality relationship is determined on three replicate batches. The results are combined to produce a log-probit dose mortality regression line from which the LC_{99} is estimated. The diagnostic concentration corresponds to twice the lowest concentration that causes 100% mortality after 60 minutes exposure and 24 h holding (or longer if indicated) of a susceptible laboratory strain or a susceptible field population or twice the LC_{99} determined in baseline susceptibility tests against a susceptible laboratory strain or a susceptible field population of mosquitoes (WHO, 2006).

{Note: To obtain the desired concentration, serial dilution may be required. In case, the stock is 95%, but the required concentration is 0.001%, it would be better to prepare 1% stock solution, which is diluted to 0.05%, and by diluting this the final concentration of 0.001% may be achieved. It is to be noted that direct dilution from 1% to 0.001% should be avoided.

Accurate weighing of a small quantity of ai, for e.g., < 10mg, on micro balance would be difficult. Hence, it is preferred to weigh approximate quantity, slightly more than the required quantity, and the weight can be adjusted to the volume of the solvent}

2.1.3. Residual efficacy on substrates

This experiment is carried out in the laboratory to select target dosages for the Phase II evaluation in experimental huts based on the residual activity of the insecticide or insecticide formulation sprayed at a range of concentrations on samples of different substrates (mud, brick, thatch, concrete, plywood etc.), which are commonly used for houses and also for experimental huts. A minimum of seven replicate blocks per concentration for each substrate are prepared, at least four for bioassay and three for initial insecticide content analysis, selected randomly.

The minimum size required for a sample of a substrate that is to be tested will be 12 cm in diameter, as the diameter of the WHO cone, which will be placed on the substrate sample, is 12 cm. Blocks of concrete, mud, plywood or thatch with 5 mm thickness are prepared in Petri dishes, and dried. The concrete and mud substrates should be left to cure for a minimum of one month before spraying to avoid extreme alkaline pH. The substrates are sprayed (all seven replicates of each substrate) with insecticide, using preferably a Potter Spray Tower®, the internationally accepted accurate method of spraying in the laboratory (WHO, 2006), to achieve a uniform residual deposit of the desired concentration of active ingredient per unit area. In case of the non-availability of Potter Tower, other types of sprayers (such as compression or track sprayers) can be used with proper calibration. All the treated substrate samples are horizontally stored unsealed in climatic cabinets under controlled conditions of 27°C ± 2°C and 80% ± 10%RH with air circulation and ambient light cycles until they are used for testing.

A minimum of one positive control, having approval of WHO PQT/VCP and a similar mode of action, will be used. If no product is available with similar mode of action, the positive control can be chosen based on manufacturer's claim (e.g., if the claim is for duration of six months residual activity, an insecticide with similar duration will be used). Negative control substrate blocks will be sprayed with water using a thoroughly cleaned sprayer.

Three treated samples of each substrate are analysed for insecticide content. The samples are labelled and packed individually in aluminium foil and dispatched to any certified /recognized analytical laboratory for insecticide content analysis.

Residual activity is determined through cone bioassays on treated samples exposing a minimum of 40 mosquitoes of the target vector species per concentration, per substrate in four replicates of 10 mosquitoes each. Non-blood-fed susceptible female mosquitoes aged 2–5 days will be released into WHO cones for an exposure period of 30 minutes. The substrate samples are kept at 30°C between bioassays. After the exposure, the mosquitoes are kept in wax coated paper cups

(150 ml), 10 mosquitoes per cup, provided with cotton pad soaked in 10% glucose solution. The cups are maintained at 27°C ± 2°C and 80% ± 10% RH for 24 h and at the end of this holding period, the mortality is recorded.

Bioassays on the treated substrate samples should be done initially for one week and subsequently at fortnightly/ monthly intervals until the mosquito mortality drops below 80% after 30 minutes exposure and 24 h holding for at least two consecutive months. The period (in months) is reported during which the mortality is equal to or greater than 80%. From this assessment, the minimum concentration that causes 100% mortality will be determined, and including this (base) another two to four concentrations (i.e., 2, 3, 4, and 5 times higher than the base) will be selected for Phase II evaluation.

2.1.4 Assessment of reproductive output

In case insecticides or insecticide formulations are claimed to cause reproductive impairment in adult females and have limited direct toxicity, the effect of such insecticides or formulations on reproductive output is assessed by exposing blood-fed females to treated substrates in WHO cones.

The mosquitoes used for the tests should be 3–8 days old and should not have previously received a blood meal. The mosquitoes should be blood-fed 2–5 h before exposure, and care should be taken to ensure that only blood-fed mosquitoes are exposed in the tests.

Mosquitoes are then transferred to individual paper cups or plastic tubes with damp oviposition papers and access to the sugar solution. Every 24 h for up to 4 days, mosquitoes are monitored for survival and oviposition and recorded.

The cumulative proportion of mosquitoes that have laid eggs and the mean number of eggs laid are estimated.

The hatchability of eggs is then measured by flooding the eggs within 24 h after laying in 200 ml of water and counting the number that hatched. The number of larvae that hatch should be recorded three days after flooding. A record of the total number of eggs laid and the total number of eggs that hatch should be made for each female.

To ensure that reductions in egg laying are not a result of general laboratory/insectary conditions, results will be discarded if less than 50% of control mosquitoes lay eggs and/or less than 50% of eggs laid by control mosquitoes hatch.

Box 2.1: Adult susceptibility test (WHO paper assay, tube method)

Susceptibility test is conducted using the WHO test kit and method (WHO, 2016). The test kit and the papers impregnated with insecticides at the WHO recommended diagnostic concentration 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:

1. Six green dotted (holding tubes), four red dotted (insecticide exposure tubes), and two yellow dotted (control exposure tubes) 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;
2. Copper and steel clips;
3. Instruction sheet;
4. Log-probit papers;
5. Report forms;
6. Glass aspirators with 60 cm rubber tubing and mouthpiece;
7. Roll of adhesive tape and
8. White paper sheets (12 x 15 cm).

Method:

- The tubes with green dot should be used for holding mosquitoes,
- The tubes with yellow dot to be used for exposure to oil-treated papers (the control), and
- The tubes with red dot should be used for exposure to insecticide papers.
- The six green dotted holding tubes should be lined from inside with plain white paper fastened with steel clips, and later fixed to the slides by threading into screw caps.
- The two yellow-dotted tubes are lined from inside with oil-treated control papers duly fastened with copper clips, and
- the four red-dotted tubes are lined with insecticide papers impregnated at diagnostic concentration and fastened with copper clip.
- It is to be ensured that the label of the papers is visible on the outside of the tube.
- Tests should be performed exposing preferably 2–5 days old glucose-fed (10% in water) females of laboratory strain or 2–5 days old glucose-fed F1 female progeny of field-collected adults or 2–5 days old glucose-fed females emerged from the immature collected from field.
- Where only field collected adults can be used, their physiological stage (i.e., unfed, blood-fed, semi-gravid, gravid) should carefully be recorded (WHO, 1998). Batches of 25 non-blood-fed female mosquitoes, aged 2–5 days, are introduced into each of the six holding tubes (green dotted) through the small orifice on the slide and closed.
- The mosquitoes are held for one hour at 27°C ± 2°C and 80% ± 10% RH to get acclimatized.
- The holding tubes are appropriately labeled, indicating the locality, species tested, etc., and provided with glucose source for feeding.
- At the end of the holding time, moribund (i.e., those unable to fly) and/or dead mosquitoes, if any, are removed from the tubes.
- The yellow-dotted tubes with oil-treated control papers and the red-dotted tubes with insecticide-impregnated papers are screwed to the respective holding tubes.
- By gently blowing, the mosquitoes in the holding tubes are transferred to the tubes with the oil-treated (control) papers and with insecticide-impregnated papers. The tubes are held vertically for one hour of exposure under subdued light.
- During the exposure time, the glucose source should be removed.
- At the end of the exposure time, the mosquitoes are gently blown back into the respective holding tubes that are placed vertically in a dark place at 27°C ± 2°C and 80% ± 10% RH with glucose solution for 24 h.
- Dead mosquitoes are counted after 24 h.
- A total of 100 mosquitoes in four replicates, 25 in each replicate (each tube), are used for each test concentration and the control in two replicates.
- Results are expressed as percentage mortality after 24 h and corrected for control mortality.
- After each exposure, the test kit should be washed with soap and clean water and dried.

Box 2.2: Adult susceptibility test (WHO bottle assay)

Currently, data on insecticide resistance are generated using the WHO tube method (filter-paper assay), with insecticide-impregnated filter-paper that tests adult susceptibility to insecticide compounds. However, there are certain issues with filter-paper assays, particularly for evaluating some new insecticide molecules having different modes of action and/or particular chemical properties; for example, the molecules cannot be dissolved in carrier oils/solvents that are presently used and/or will be unstable on Whatman® filter-paper No.1 while impregnating. Therefore, it was suggested to complement filter-paper assays with the CDC/WHO bottle assays for the detection of insecticide resistance in vector populations and to ensure reproducibility of successive tests and more reliability of the results (WHO, 2016). In general, CDC bottle assay measures the length of time to knock down (or incapacitate) the mosquitoes and does not provide mortality at 24 h (or more) post-exposure for most of the compounds. However, mortality (24 h post-exposure or more, if relevant) will be the primary endpoint (ideally with 1h exposure time to the insecticide) in the WHO bottle assay. These assays would standardize determining discriminating concentrations and exposure times for particular insecticides and for vector species using populations known to be susceptible.

A detailed description of the CDC bottle bioassay, including the methodology, was published by the CDC in 2010. Both WHO and CDC bottle assays can reliably be used to detect insecticide resistance in vectors, but the CDC assay results are not directly comparable with those obtained from the WHO paper assay in tube test, as the endpoint measured in the CDC method is the proportion of mosquitoes knocked down or incapacitated. Whereas, the WHO assay measures mortality of mosquitoes.

Like the WHO paper assay, the CDC bottle assay is performed on adult females collected from the field or on those reared in insectary from larval collections.

Bottle bioassays will be performed using a 250 ml Wheaton® bottle coated with one ml solution of carrier/solvent (acetone/ 81% rapeseed oil methyl ester + acetone) containing the insecticide at diagnostic concentration. Four replicates of diagnostic concentration, each containing 25 non-blood fed, 3–5 days old mosquitoes, and two replicates of control bottles (solvent only) with 25 mosquitoes each will be prepared.

The number of knocked down mosquitoes will be counted and recorded at a constant interval of time (e.g., every 5 minutes for fast-acting insecticides) to 1 h of exposure (or more if required). Then, the mosquitoes will be transferred into insecticide-free holding cups covered with a netting piece and provided with a cotton pad soaked in a 10% glucose solution on top. The number of dead mosquitoes will be recorded at 24 h (or more if required).

The mosquitoes in holding cups will be maintained at 27 ± 2°C and 80% ± 10% RH.

- To record the sterilizing effect (reduction in fecundity and hatchability) of the insecticide (e.g., Pyriproxyfen), in addition to mortality in bottle bioassay, female mosquitoes will be blood-fed before bioassays (i.e., before exposure to insecticide) and maintained under observation over several days to record the number of eggs laid, and the number of larvae emerged in comparison to an unexposed (control) batch.

The bioassay will be repeated three times using different batches of mosquitoes and bottles. Every time, 100 mosquitoes in four replicates will be tested with parallel control in two replicates of 25 mosquitoes each.

Mortality, Calculations and Corrections:

The number of dead mosquitoes in the exposure and the control tubes/bottles are recorded at the end of the specified post-exposure holding period. The test/control mortality is calculated by summing the number of dead mosquitoes across all test/control replicates. This is expressed as a percentage of the total number exposed in test/ control.

$$\%\ \text{Test/Control mortality} = \frac{(\text{Total number of dead mosquitoes})}{(\text{Total number exposed})} \times 100$$

If the control mortality is $<5\%$ (i.e., one dead out of 25 mosquitoes), correction of test mortality is not required, whereas mortality between ≥ 5 and $\leq 20\%$ needs correction with Abbott's formula (as given below) and if the control mortality is $>20\%$ the test is to be discarded and repeated.

Abbott's formula as given below:

$$\%\ \text{Corrected mortality} = \frac{(\%\ \text{Treated mortality} - \%\ \text{Control mortality})}{(100 - \%\ \text{Control mortality})} \times 100$$

Interpretation of results:

Corrected mortality: 98 – 100% = Susceptible

90 – 97% = Possible resistance; to be verified by additional tests or molecular assays.

<90% = Resistant; additional tests **are not required**, provided at least 100 mosquitoes of the target species were tested.

Intensity bioassay: If the mortality is <90% by testing 100 mosquitoes, it is necessary to test additional mosquitoes at 5x and 10x discriminating concentrations following the same procedure described above.

Interpretation of results from the intensity bioassays (WHO, 2016): 98-100% mortality at 5x concentration indicates a low resistance intensity, and further testing at 10x concentration is not necessary.

Mortality of <98% at the 5x concentration indicates a moderate resistance intensity. It is recommended to assay further at the 10x concentration.

Mortality between 98 and 100% at the 10x concentration confirms a moderate resistance intensity.

<98% mortality at the 10x concentration indicates a high resistance intensity.

If resistance is confirmed at 5x and especially at 10x concentrations, operational failure is likely.

2.1.5. Irritant or excito-repellent properties

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

The insecticide irritancy is first assessed by exposing mosquitoes to filter-paper impregnated with technical grade of the given insecticide at diagnostic concentration. Determination of diagnostic concentration is described in section 2.1.2. The method of assessing irritancy is the same as described below for the treated substrates. 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 by exposing mosquitoes to different substrates (commonly used for making houses/shelters such as mud, cement/concrete, plywood, thatch) treated with the given insecticide or the insecticide formulation.

The substrates are sprayed with the recommended concentration (i.e., the lowest concentration that causes >80% mortality for a longer duration) of the given insecticide (refer to section 2.1.3). For each substrate, 50 susceptible, non-blood-fed, 2-5 days old female mosquitoes are individually introduced into plastic cones. After allowing 60 seconds for the mosquitoes to settle down, the time elapsed between the 'first landing' on the substrate 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 FT_{50} and FT_{95} (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, especially when new molecules are tested. For positive control also, 50 mosquitoes will be individually tested per substrate.

Using log-dose probit regression analysis, the relationship between dose and percentage taking off due to irritability can be understood.

2.1.6 Cross resistance

Mosquitoes develop resistance to a given class of insecticide e.g., DDT (organochlorine) or deltamethrin (synthetic pyrethroid), on continuous exposure to the same. Whereas, in cross-resistance, mosquitoes resistant to a given class of insecticide also exhibit resistance to insecticides of other class to which they had no exposure at all. Cross resistance phenomenon in mosquitoes has significant operational implication. Cross resistance is determined using WHO filter-paper/bottle assay method, exposing the target mosquito species to the diagnostic concentration of the insecticide of interest as described in Box 2.2 & 2.3 (Section 2.1.2). The test procedure is same for determination of resistance and cross-resistance. The mosquito strains used for the assays should be susceptible to the candidate insecticide being tested. However, for insecticide formulations with claim for resistance management, the mosquito strains used for the assays should be resistant to the said candidate insecticide.

The susceptibility/resistance status of vector species should be categorized as per the WHO criteria: susceptible: 98 to 100% mortality, possible resistance: 90 to 97% mortality, resistant: <90% mortality. Data should be recorded in the format given in Table 2.1.

Table 2.1: Insecticide susceptibility test (WHO paper assay, tube method)

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

Insecticide (%) .....Impregnation date .....No. of times paper used .....

Date of Test.....Temp: Min ..... Max .....Humidity: Min .....Max.....

Test species .....Lab/F1/Field collected .....

Exposure: Start time ..... End time.....Holding: Start time..... End time .....

Test done by..... Supervised by.....

Replicate	No. exposed*	No. knocked down in 1 h	No. dead after 24 h	% Mortality	% Corrected mortality#	Remarks
Treated 1						
Treated 2						
Treated 3						
Treated 4						
Treated total						
Control 1						
Control 2						
Control total						

*25 mosquitoes per 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).

$$\% \text{ Corrected mortality} = \frac{(\% \text{ Treated mortality} - \% \text{ Control mortality})}{(100 - \% \text{ Control mortality})} \times 100$$

2.2. Phase II – Small-scale field evaluation (in experimental huts)

Objectives

General

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

Specific

- 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 determine the optimum application dosage of the insecticide to be used for Phase III evaluation.
- To record the ease of application and perceived side-effects by the spray-men and the inhabitants during application and use.

2.2.1. Evaluation in experiment huts

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.

Duration: 12 months

Number of dosages to be used: 3–5

2.2.1.1 Experimental hut design

The experimental hut consists of a single room with four windows; the size of each window should be 0.45 x 0.45 m, grilled with wooden planks fixed horizontally in a 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 (Figure 2.1a). There are two windows on the front door side, one on each side, and a screened (using nylon mesh) verandah (verandah trap) at the rear side (Figure 2.1b). The hut with dimensions resembling those of the village huts (length 3 m, width 3 m, and height 2.5 m), has brick walls with cement plastering and a thatched roof, above which there is tin-sheeted roofing for protecting the thatched roof (Appendix 1). There should not be space between the thatched ceiling and the tin-roof. The hut is 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 two feet away from the hut walls, except on the back side of the hut where it is 1.5 ft away from the base of the verandah trap. At the centre of the hut, the roof is at the height of 2.5 m and near the wall, the height is 2 m; this height difference is to maintain a slope of the roof. The eave on the rear side (facing 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 0.75 m x 1.5 m facing the west (Figure 2.1a).

For small-scale field trials, preferably, several huts are required to compare different dosages or treatments simultaneously. A minimum of four replicates (four huts) per treatment and an equal number of control huts are to be used.

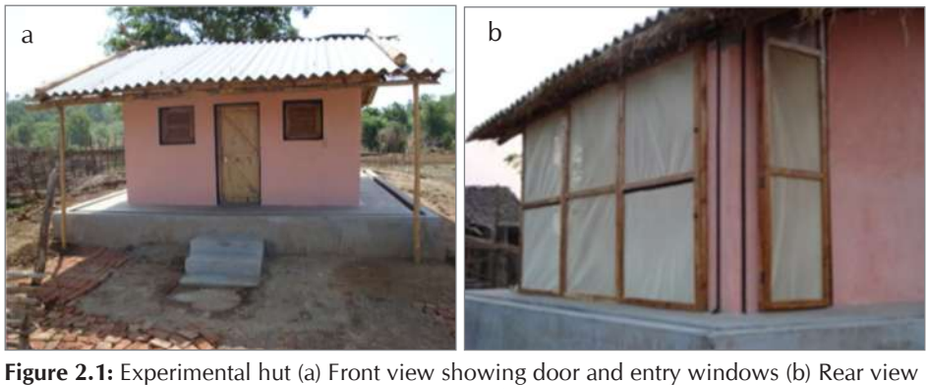

Figure 2.1: Experimental hut (a) Front view showing door and entry windows (b) Rear view showing verandah trap

2.2.1.2. Assessment prior to hut trial

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

(*The study duration may be extended if the number of mosquitoes of the target species collected in the control arm is found to be too low to perform statistical analysis or the effective duration of residual activity of the product is claimed/ observed to be longer; this will be done with administrative and sponsor's approval*)

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 15 days prior to assessment of hut suitability.

Hut suitability: Subsequently, the suitability of the experimental huts for conducting the trial is assessed over one-month prior to the start of the trial, based on the following criteria.

Indoor resting of mosquitoes: The resting mosquitoes are collected from the experimental huts in the morning hours, twice weekly, keeping equal intervals between the two successive collections. In parallel, mosquitoes should also be collected from the randomly selected village huts (the 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 (Table 2.2). 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 huts.

Tightness of huts (from recovery rate): Recovery rate (number of mosquitoes re-captured/ total number released) × 100 is used to verify the tightness of the experiment huts. Around 75 (depending on availability) fully-fed field-collected female mosquitoes of the target vector species 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.

Absence of scavengers: To ensure the absence of scavengers inside the experimental huts, four batches of 25 dead anopheline mosquitoes are kept on the floor, including verandah (in four corners) of each hut in the evening and the number present the next day morning is recorded. Such observations should be made eight times, twice a week, during the four weeks.

The experimental 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.

The Phase II experimental hut trial for evaluating an insecticide (candidate product) for IRS will conventionally have a negative (untreated) and a positive control (reference). The negative control involves only a sleeper without insecticide treatment or with the formulation minus the active ingredient or distilled water. Positive control will be an insecticide commonly used by the national programme. Since the candidate products show significant variations in their design/formulation; it has become important to ensure a comparable performance of the candidate product (second-in-class product#) to a comparator (active comparator/ first-in-class product#). Thus, by comparing with an active comparator/ first-in-class product whose public health value (epidemiological efficacy) has already been demonstrated, there won't be any need for epidemiological impact assessment for each candidate product. Therefore, the WHO has recommended a non-inferiority trial design (WHO/CDS/GMP/2018.22. Rev.1.) for Phase II evaluation of candidate IRS products using a defined set of entomological parameters.

2.2.1.3. Non-inferiority trial

A non-inferiority trial aims to assess that the new/candidate product is not inferior to the comparator by more than a small pre-determined margin (EMEA, 2005), which is called a 'non-inferiority margin'. In non-inferiority design, new products are expected to be compared directly to the first-in-class product, which is referred to as the 'active comparator' and such comparisons require strong power calculations. WHO has worked out an acceptable level of non-inferiority margin. Also, there should be sufficiently large sample sizes to confirm that the efficacy of the

Table 2.2: Comparison of Anopheline Density between Experimental and Village Huts (for assessment of hut suitability)

Place of collection:.....Name of study site: Village.....District:.....Date of collection: .....

..... Man-hour spent in each hut: .....

Expt. Hut No.	No. of anophelines collected		No. of mosquitoes of the target vector species collected		PMD (♀)		Village Hut No.	No. of anophelines collected		No. of mosquitoes of the target vector species collected		PMD (♀)	
	♂	♀	♂	♀	Anophelines	Target species		♂	♀	♂	♀	Anophelines	Target species
Total/ Average													

new product is within the acceptable margin. Thus, the non-inferiority margin, the efficacy of the active comparator, the characteristics of the test site and the comparison method will decide the sample sizes (WHO, 2019).

It is recommended that the active comparator should, preferably, be a first-in-class product that exhibited its public health value through cluster randomized controlled trials with epidemiological endpoints and not a second-in-class product. However, in case of difficulty in getting a first-in-class product for comparison, a second-in-class product may be used as the active comparator provided it is non-inferior to the first-in-class product and pre-qualified. If two or more products with same epidemiological efficacy are available in the same class, anyone, having the required epidemiological data, can be chosen, although the selection needs to be justified.

In case a new product is claimed to be superior to the current standard of care (intervention/product currently in use in the programme, belonging to another/older class), then it is necessary to demonstrate that the new product is superior, in terms of efficacy, over the intervention/product currently in use (standard comparator), based on entomological endpoints (WHO, 2019).

Primary endpoints

The primary endpoint for the non-inferiority IRS trial will be mosquito mortality (not corrected for the negative control). Mosquito mortality is normally recorded at 24 h unless it is specified with a justification to record mortality up to 72 h (depending on the mode of action of the candidate insecticide). To be defined as non-inferior, a new IRS product must show non-inferiority to the active comparator in terms of mosquito mortality alone.

Secondary endpoints

Besides the primary, the following secondary endpoints should be measured:

a) percentage of blood-fed mosquitoes alive (95% confidence interval [CI] estimates)

b) induced exophily (collected from exit/veranda trap) (95% CI)

c) deterrence (preventing hut entry) (95% CI)

d) personal protection (95% CI)

e) residual efficacy in weeks/months, measured by cone bioassay of free-flying mosquitoes over time until mortality falls below 80%

Additional endpoints can be included as per manufacturer's claim, such as reproductive effects. Also, depending on the insecticide under investigation, mortality is recorded after 24, 48, or 72 h.

{In order to be a part of the intervention class covered by a WHO policy recommendation, a new/candidate product must demonstrate non-inferiority to the active comparator and superiority over the control (or the current standard of care) with primary endpoint(s)}.

#First-in-class refers to the first product with a novel entomological effect (e.g., reducing human–vector contact, or decreasing vector survival, biting rates or susceptibility to infection or transmission), the public health value of which is ascertained by the WHO Vector Control Advisory Group (VCAG) based on the demonstration of its entomological and epidemiological efficacy against vectors and human infections and/or disease, respectively. Once the public health value of a first-in-class product has been ascertained, a WHO policy recommendation will be issued, establishing a new intervention class. Subsequent products that demonstrate the same entomological effect as the first-in-class products are referred to as "second-in-class" products. Candidate second-in-class products are not required to provide epidemiological data for their assessment. Instead, they are assessed by the WHO Prequalification Unit (PQT) based on their safety, quality and entomological efficacy data (WHO, 2017a) (definition taken from WHO/CDS/GMP/2018.22. Rev.1.)

Non-inferiority margin

An odds ratio of 0.7 has been estimated to be the non-inferiority margin between the active comparator and the candidate/ second-in-class product.

The odds ratio is estimated using logistic regression if the primary endpoints are dichotomous variables i.e., a mosquito is either dead or alive, fed or unfed.

When the primary endpoint is mosquito mortality, the non-inferiority margin is set at 0.7, as higher mortality indicates a better product. A candidate/ second-in-class product is shown to be non-inferior in terms of mosquito mortality if the lower 95% CI is greater than 0.7. An odds ratio of 0.7 equates to a difference in percentage mortality of no more than 9% (WHO, 2019).

Study arms

Inclusion of study arms depends on whether or not there is a WHO-prequalified product of the same insecticide class.

If a pre-qualified reference product of the same insecticide class is available, the following will be the study arms:

1. Water (negative control)@
2. WHO-prequalified IRS product of the same insecticide class as the candidate IRS product (active comparator: this can be a first-in-class or second-in-class product)
3. Candidate second-in class IRS product (test product)

If there is no pre-qualified reference product of the same insecticide class, the trial should include a minimum of four study arms as given below:

1. Water (negative control)@
2. Standard comparator* (Standard-of-care IRS product used in the study area or IRS product with similar residual activity to the candidate IRS product)
3. First-in-class product (WHO-prequalified IRS) of a different insecticide class, but with a similar expected duration of residual efficacy (active comparator) and for which data demonstrating epidemiological impact are available
4. Candidate second-in class IRS product (test product)

Additional arms may include the insecticide classes currently used or proposed to be used for IRS in the country.

Since experiment arms cannot be rotated in the IRS trial, it is recommended to use four huts per treatment arm to overcome the spatial heterogeneity between huts and to improve data quality, and power analysis should consider this at the beginning of the study. In order to spray an adequate number of huts with each intervention, the study arms should be planned to an optimum number in each study site. In case the number of huts is a limiting factor in a trial site, the number of negative control huts can be reduced to one.

2.2.1.4. Rotation of sleepers

For IRS trials, treatments cannot be rotated; hence it is necessary to demonstrate that there is little or no variation in the attractiveness of huts during a pre-trial assessment. The sleepers should be rotated between huts so that every sleeper is allocated to each hut-treatment an equal number of times. Thus, the sleepers will have to be rotated daily between the huts.

(It is to be noted that an insecticide class is different from an intervention class: an intervention class is established based on the evidence of epidemiological impact of a first-in-class product and may include IRS products of different insecticide classes: WHO, 2019).

2.2.1.5. Ethical clearance and insurance coverage

The trial proposal should be submitted to the human ethics committee of the respective institutions and authorities and clearance should be obtained before undertaking the trial. Informed consent, prepared as per the National Ethical Guidelines for Biomedical and Health Research involving Human Participants by ICMR (Annexure 1), should be obtained from the volunteers involved in the study (Annexure 2). Proper medicare, including chemoprophylaxis (as per the national guidelines), should be given to the volunteers. Further, the human volunteers participating in the study should be insured by the Sponsor/Funding Agency to meet the financial compensation for death and physical disability and mental shock arising out of the trial and also treatment and hospitalization expenses arising out of illness consequent to the trial, as recommended by the ethics committee.

2.2.1.6. 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 at the trial site, and a responsible person should have access to them (Box 2.4).

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 on the fresh surfaces.

The insecticide formulation should safely and correctly be applied in the huts following the WHO guidelines (WHO, 2003) (Box 2.5). 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, removing and replacing the door, substrates and ceiling material between trials will be necessary. Alternatively, hardboards/plywood can be used with a coat of mud for walls and a layer of paddy straw for the roof. Once the trial is over, the hardboards/plywood should be removed from the huts and fresh materials should be used for evaluating the next product. It is important to ensure that there should not be any contamination from the previous insecticide evaluated in the huts.

2.2.1.7. Assessment of spray quality

To assess the quality of spraying, at least four Whatman® filter-papers (each paper 15 cm x 15 cm) leveled properly will be struck on the walls (one on each wall of the hut) of each experimental

@ Negative control arm: A negative control arm (spray of untreated water) should be included in all Phase II trials of IRS products to ensure adequate quality of the trials in experimental huts and to estimate natural mortality, blood-feeding, and deterrence, which are the secondary endpoints required for assessing the efficacy. If the overall 24-hour mortality in the control arm is >10% over the study period, the trial needs to be examined and repeated.

**Standard comparator: If the candidate/ second-in-class or the first-in-class product is claimed to be superior to the product currently used for IRS in the country (current standard of care, which belongs to another/older class), where the trial is proposed to be carried out, then a standard comparator (current standard of care) is necessary to be included in the trial. It should be shown from the study that the candidate product performs significantly better (superior) than the standard comparator. The standard comparator can also be a WHO-prequalified product (though not a pyrethroid insecticide) used in the region.*

As per the WHO guideline, if a candidate or a new product is not shown to be better than the current standard of care, it is still essential to prove that it is non-inferior to the standard of care (which will be the active comparator) and superior to the negative control arm (untreated water) of the trial (this is to prevent candidate second-in-class products from being evaluated in sites where first-in-class products are no longer fully effective).

hut before spraying and removed after complete drying. The papers will be wrapped in aluminum foils and subjected to analysis for insecticide content by a GLP-certified laboratory. The chemical analysis results are combined for each substrate to provide the average insecticide concentration (in mg/m^2).

2.2.1.8. Evaluation

2.2.1.8.1. Dosage determination and residual activity assessment

The residual activity of the target dosages (as determined in the Phase I trial) is assessed by conducting bioassays at regular intervals, preferably on day 1, day 7 post-spraying and after that weekly/ fortnightly, using WHO cones. Batches of 10 non-blood-fed, 3–5 days old mosquitoes are released into each cone and exposed for 30 minutes on each hut's walls and ceiling. Wherever it is not practical to use non-blood-fed 3–5 days old (F_1 females) mosquitoes for the assay, wild-caught female mosquitoes may be used, recording their gonotrophic conditions.

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

When selecting a dosage for testing, safety issues need to be considered.

2.2.1.8.2. Air-borne toxicity of the 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 h up to a maximum of 12 h 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 into 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 h observation and mortality, if any, is recorded. The air-borne toxicity is assessed by comparing the treated mortality in comparison to the controls.

2.2.1.8.3. Efficacy and impact on vector behaviour

When the trial is conducted in experimental huts, the volunteers involved in sleeping in the huts should carefully follow the instructions given by the research team supervisor. The sleepers should follow a standard 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 check at night (without intruding in to their privacy) to ensure that the instructions are being followed by the volunteers sleeping in the huts. The volunteers will be asked not to smoke or to make fire inside the hut. While the volunteers are inside the huts for sleeping, it should be ensured that the windows are kept open.

Mosquito collections

Huts will be sprayed in the morning hours, ~7 am, and the sleepers will enter the huts in the evening, ~7 pm, i.e., ~12 h after spraying. In the evening, before the volunteers occupy the hut, its room and verandah should be cleaned, and white cloths spread on hut floor, including the verandah. The verandah trap is furnished with cotton pads soaked in 10% 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 inside net (if present) using aspirators and flashlights (Figure 2.2). 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 10% glucose solution, are kept on observation under controlled conditions (temperature: 27 ± 2°C and RH: 80 ± 10%) for 24 h to record delayed mortality, if any.

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

Figure 2.2: Mosquito collection inside an experimental hut

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.

2.2.1.8.4. 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 in 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 side-effects perceived, if any, by them. In case of any such complaint, the researcher should immediately attend to it and ensure that they are relieved of the discomfort.

2.2.1.8.5. 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 entry rate is the total number of female mosquitoes collected in the hut and verandah. Certain types of repellent insecticide possess a deterrent effect causing a reduction in 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 compared 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 reduced 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 h 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 a considerable deterrent effect preventing a significant number of mosquitoes from entering the hut, the values obtained from proportions of 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 by thereduction of the number of blood-fed mosquitoes in the sprayed hut compared to the number blood-fed in the control hut. The following formula is used to calculate the personal protective effect (WHO, 2006):

$$\text{Personal protective effect (feeding inhibition) (in\%)} = 100 \times \frac{(Bc - Bt)}{Bc}$$

Where, Bc is the total number blood-fed in the control hut and

Bt is the total number blood-fed in the sprayed hut

Similarly, the overall insecticidal effect of spraying should consider 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):

$$\text{Overall insecticide effect} = 100 \times \frac{(Dt–Dc)}{Ec}$$

Where, Dt: total number of mosquitoes dying in the sprayed hut,

Dc: total number dying in the control hut and

Ec: 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.

Generalized linear regression models (GLMs) are recommended to relate the outcome variables to the intervention and covariates. Depending on the endpoint(s) under study, the model will be chosen. For binary endpoints, such as the proportion of mosquitoes dying or feeding or exiting, a logistic model is appropriate. For count data, such as the number of mosquitoes entering a hut, a Poisson or negative binomial model may be more appropriate. Huts, sleepers and time since spraying for IRS will be included in the models as fixed or random effects. The intervention itself should be included as a fixed effect. All covariates should be categorical and the active comparator should be kept as the reference category (intercept). The estimated effect of the intervention and 95% CIs should be reported in all instances.

Non-inferiority test

The candidate/test product is regarded non-inferior if:

1. The lower 95% CI estimate of the odds ratio describing the difference in mosquito mortality between the candidate and active comparator product is greater than 0.7 and,
2. The upper 95% CI estimate of the odds ratio describing the difference in mosquito blood-feeding between the candidate and active comparator product is greater than 1.43.

(The decision whether a product should achieve either or both of the primary endpoints will be made by WHO considering the current understanding of the primary entomological efficacy of the first-in-class product)

In the sites where the experimental hut trials are carried out, the resistance status of the target mosquito species should be characterized. This will include the determination of resistance frequency and mechanisms, especially biochemical mediated ones (WHO, 2016). In addition, in areas where mosquitoes may feed outdoors on cattle but enter huts to rest, the mosquitoes collected from human-baited huts should be identified to species, with a screening of the blood meals for a source of feeding to determine the proportion that fed on humans. This will help to list the species present and their human blood index, preferably within a few weeks of the trial, as mosquito diversity may change over time.

Superiority test

The candidate product will be considered superior to the negative control or standard comparator in terms of mosquito mortality if significantly higher mortality is recorded at the 5% significance level (i.e., p-value < 0.05) *(can be estimated from the logistic regression by comparing models that do and do not differentiate between the candidate and control / standard comparator using a likelihood ratio test)*. The mortality endpoint is used for evaluation.

Box 2.3: Safety measures (Source: Malaria Vector Control WHO/WHOPES/2002.5 and Environmental Codes of Practice (ECoP) - NVBDCP (http://nvbdcp.gov.in/ECoP.html)

Spraymen

- All spray personnel should wear appropriate personnel protective equipment (PPE) such as goggles, gloves, boots and two sets of working clothes during the entire spray operation.
- Spraymen 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
- Spraymen should be advised to take a bath after each day's work.
- Spraymen should ideally work for 5–6 h 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, it 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 h in severe cases.

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 the occurrence of convulsions after severe intoxication, intravenous injection of 5-10 mg Diazepam (or other benzdiazepine derivatives) should be given.

Box 2.4: General specifications of spray pump and spraying

(Detailed specifications of the spray pumps are given in the WHO-Equipment for Vector Control, Specification Guidelines, 2nd Edition, 2018 and in NCVBDC- Manual on Integrated Vector Management in India, 2022)

Stirrup pump (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].
- To get this discharge rate, 20–26 strokes per minute are required with 10–15 cms plunger movement at a pressure of 10 psi.
- Distance and angle of lance from wall: 45 cm and 60°.
- Spray is applied in vertical swath of 53 cm (21 inches) wide and successive swaths should overlap by 7.5 cm (3 inches).
- Rate of coverage: 5 min per house with an average sprayable surface of 150 m^2

Hand compression sprayer:

- The maximum operational recommended tank pressure is 55–58 pounds per square inch (P) (~ 400 kPa or 4 bar).

- This pressure is attained with no more than 60 full strokes of the pump.
- The hose shall be at least 1.2 m long with an inside diameter of not less than 6.0 mm.
- The length of the lance attached to the trigger valve shall be at least 500 mm and fitted with a control flow valve to ensure spray is delivered at a constant flow rate as tank pressure decreases when spray solution is exhausted.
- A constant flow at 1.5 bar (21.8 psi) is recommended and it is achieved by fitting a red colour 1.5 bar control flow valve (CFV) (WHO, 2015).
- Use of 8002E Flat Fan Spray Tip Nozzle is recommended for IRS, and when operated at 1.5 bar pressure, the discharge rate shall be 550 ml/min.
- Previously, the WHO recommended the fitting of hardened stainless-steel nozzles, but presently, ceramic nozzle tips are found to be more durable and thus recommended.
- Swath size: 75 cm with 5 cm overlap.

Calibrating the sprayer nozzle

- The nozzle of the sprayers to be calibrated with water in the tank as follows:
- The sprayer is pumped to ensure working pressure is reached (4 bar or 58 psi);
- The trigger or on/off valve is opened for 1 minute, the discharge is collected and the amount is measured in a measuring jar; and
- It is repeated three times and the average discharge per minute is calculated.
- The correct discharge of an 8002E nozzle at 1.5 bar CFV or 22 psi pressure is 550 ml per minute (the correct discharge of an 8002E nozzle).
- Nozzle tips are considered worn if the flow rate exceeds the rate of a new tip by 10%. Therefore, based on a pressure of 1.5 bar, a discharge of 550 ml is normal for an 8002E nozzle, and between 550 ml and 605 ml means worn but serviceable, but if the flow rate is >605 ml, the nozzle should be discarded and replaced (WHO, 2015).

2.3. Phase III – Large-scale (village-level) field evaluation

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

Duration: 18 months

Objectives

1) To establish the efficacy of insecticide formulations at the selected dosage against the target vector species (when sprayed in all or most households in the community) in terms of changes in exiting behaviour, indoor and outdoor resting densities, human landing densities and probability of daily survival after spraying.
2) To compare the above parameters between arms.
3) To confirm residual activity and application intervals.
4) To study the impact on disease incidence/ prevalence.
5) To assess community perception and acceptability of the new insecticides or formulations and collateral benefits.
6) 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.

2.3.1. Selection of villages and collection of baseline data

Phase III trials are usually designed as cluster randomized trials (CRT); the unit of intervention under this phase is the village. In CRT, groups of individuals, for example, a household, village, or larger geographical area, are randomly allocated to receive either intervention/treatment

(indoor residual spraying with the candidate insecticide) or control (comparator/positive control) (WHO, 2017a). Cluster randomized trials are useful when contamination is likely to occur in comparisons of vector control interventions within the same community. In addition, CRT can have practical advantages of lower implementation costs or administrative convenience. For details of conducting CRT for Phase III evaluation, the WHO guideline on "How to Design Vector Control Efficacy Trials", Guidance on phase III vector control field trial design provided by the Vector Control Advisory Group, WHO/HTM/NTD/VEM/2017.03, may be referred to.

Phase III trial can be done in areas where the ongoing IRS operation can be suspended during the trial period. For comparison, a WHO-recommended IRS formulation with a similar mode of action or the conventional formulation of the same class of insecticide, which has been used by the (local) control programme, should be included as a positive control. The selection of a suitable positive control can be made according to the claim of the manufacturer (for example, if the candidate insecticide is claimed to have a residual activity for 6 months, an insecticide with a similar duration should be used).

Phase III IRS trials of new formulations should at least make a comparison with negative control (i.e., existing LLIN coverage) and ideally with an active comparator [first-in-class product that demonstrated its public health value by means of cluster randomized controlled trials with epidemiological endpoints or a second-in-class product that has been shown to be non-inferior to the first-in-class product and pre-qualified (WHO, 2019)] arm of a WHO PQT-VCP listed or an IRS product/ formulation already registered with CIBRC of India.

Power calculations should be done in consultation with a statistician as part of any trial protocol to ensure sufficient, but not wastefully excessive, power. The power will depend on the sample size, the expected effect size, the amount of variability in the response variable and the significance level. At least 80% power should be aimed. It is recommended that a minimum of eight clusters per arm be used to minimize imbalances between study arms.

The selection of villages for the trial should be made in consultation with the state/ district health officials. The villages selected for spraying and comparison (comparator/positive control) should be eco-epidemiologically homogenous. Villages with an average annual parasite incidence (API) of >2 (in the last 3–5 years) with a minimum population of 3,000 (8–10 villages), each for treatment and comparison, should be selected for the trial. Selection criteria can also include population size, ease of all-weather access for vector collection, sizeable vector populations, and absence of indoor residual spraying (IRS). In cluster-randomized trial, for testing different arms of insecticides/ insecticide formulations for IRS, it would be ideal to select isolated villages (clusters), in terms of the human population, breeding habitats and environment, to avoid spill over or infiltration of mosquitoes from outside. The distance between villages should be wider than the known or expected flight range of the vectors; thus, it should be at least 500 m to prevent mosquito movement between sprayed and comparison areas. The selected villages (clusters) should be randomly allotted to treatment and comparison (positive control) arms to reduce the selection bias and 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), mosquito species composition, vector density, types of breeding habitats, LLIN usage, human biting rates, sporozoite rates and incidence of disease. The required baseline data for such stratification should be collected prior to the trial and comparable treatment and positive control groups selected. Collection of baseline data requires a preparatory phase of at least one high transmission season (3–6 months), according to the entomological and transmission patterns of the area. Failure to collect sufficient baseline data to show that treatment and comparison villages are comparable before spraying may invalidate any subsequent findings. Following the stratification, within each identified stratum, villages are randomly allocated to the treatment or comparison arm (WHO, 2006).

Also, the villages can be stratified in pairs (Matched pair designs) and from each matched 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 cluster and will determine the power of the study to detect an expected or minimum percentage impact. In the villages, the houses may vary greatly in their attractiveness to mosquitoes, hence different houses (randomly selected) should be used for entomological monitoring for each survey. Also, for practical reasons and consistency, fixed catching stations (same houses) should be monitored throughout the study period (WHO/CDS/NTD/WHOPES/ GCDPP/ 2006.3).

Thus, monitoring (mosquito collections) will be done in fixed as well as in randomly selected houses for each survey. Conducting large-scale IRS field trials with negative controls (unsprayed) is not acceptable for ethical reasons.

2.3.2. Census

In collaboration with the respective Primary/ Community Health Centre (PHC/ CHC) 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 given in Table 3.

2.3.3. Ethical considerations

For conducting the trial at the village level, necessary ethical clearance should be obtained from the ethics committees of the respective institutions/ authorities. The informed consent form and the information sheet containing the details of the trials (prepared as per the National Ethical Guidelines for Biomedical and Health Research involving Human Participants by ICMR, 2017; Annexure 1) to be provided to the households in the selected villages should also be approved by the ethics committee.

The households 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. They should also be told they have every liberty to participate or refuse to participate. Written consent will be obtained from all households (heads of the households) included for spraying under the study.

2.3.4. Spraying of villages

In the treatment villages, the candidate insecticide or insecticide formulation is sprayed, while in the comparison villages, the insecticide that is selected as the comparator/ positive control (preferably the insecticide which is in use in the programme or WHO-Pre-qualified IRS formulation of the same insecticide) will be sprayed. Spraying of insecticides in the villages is done in collaboration with the respective PHC/CHC Medical Officer and the District Medical/Health/Malaria Officer. All human dwellings, mixed dwellings and domestic animal/ cattle sheds will be sprayed to ensure total coverage. Similarly, to achieve complete spraying, all sprayable surfaces (except the floor) of the dwellings will be sprayed with a 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, spraying should be repeated.

Supervised spraying with the technique and the equipment, preferably hand operated compression sprayer (with red colour control flow valve), which are used in the routine vector control programme, will be done following the NCVBDC guidelines. The pumps will be calibrated as per the prescribed method to obtain an appropriate discharge rate for good spray. Inhabitants of the selected villages will be informed in advance of the necessary preparation to get their dwellings sprayed on the scheduled date and the benefits they would get from spraying. Necessary precautions will be taken for the protection of spray-men by providing personal protection equipment (refer

Table 2.3: Record of census of households

Village ..... Sub-Centre ..... PHC/CHC..... District.....

State..... Date of Survey..... Survey done by.....

Supervised by .....

Sl. No.	House No.	Name	Relation	Age/ gender	Education	Profession	Type of structure	No. of rooms	No. of cattle sheds	No. of temporary sheds	Sleeping Indoor /outdoor
01											
02											
03											
04											
05											
06											

to Box 2.4). Each house will be marked on the wall indicating the date and coverage of spraying [Number of rooms sprayed/ number of rooms in the dwelling (e.g., Dwelling No. 5/5)]. Spray coverage should be recorded concurrently during the spray operation, as indicated in Table 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 spray men, insecticide handlers, household members sleeping in the sprayed houses and other persons (stencil writer, supervisor) involved in the trial, and respond to any adverse health event. Further, if anybody reports with any adverse effect/ inconvenience during or after spraying should be attended to for necessary medical care. Parents of the households in the sprayed villages should be cautioned about the risky situations involving children and domestic animals with needed precautions to be taken to avoid adverse health events.

2.3.5. Assessment of spray quality

The spraymen should be trained; sprayers need to be calibrated to maintain an appropriate discharge rate and the spray operation should be closely supervised in order to achieve a quality spray (uniform application of insecticide with adequate dosage). Whatman® No. 1 filter-papers are fixed on the walls of the houses selected randomly (three sampling spots, one from roof and two from walls, in each of the minimum 10 houses selected per arm) and removed after spraying. The filter-paper samples are sent to a GLP-certified laboratory for insecticidal content analysis. Spray quality is expressed as a percentage of holdings (based on 3 sampling spots in each selected house) with adequate active ingredient content per m^2. For chemical analysis, filter-paper samples are preferred to the scrapings of sprayed mud surfaces because of the difficulties in standardizing scrapings.

2.3.6. Assay for residual activity

Residual activity of the insecticide is determined fortnightly through cone bioassays (WHO, 1981a) on different sprayed surfaces such as cement walls, mud walls, thatch, etc. in the villages. The houses having different sprayed surfaces should be selected for cone bioassays. Similarly, surfaces should be identified in comparator/positive control houses. On the selected surfaces, an area of 1 sq. ft. should be marked with a pencil/chalk. At least 4 squares should be marked for a given insecticide/ formulation for each type of surface. Not more than 2 squares are 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. In case unsprayed surfaces are unavailable in the villages, 1 sq. ft. hard boards can be fixed on sprayed surfaces and bioassays carried out with adequate replicates. Care should be taken to mark the squares at different heights on the walls. The bioassays should be done on day 1, day 7 post-spraying and thereafter fortnightly using WHO cones until mosquito mortality drops below 80%. Whenever the mortality will drop below 80% in a village, bioassays should be performed a week later to confirm the low performance. When the mortality in a village or a specific substrate will be lower than 80% on two consecutive tests, further testing should be stopped and inform the State to include under regular spraying/ intervention.

Ten non-blood-fed F1 females (3–5 days old) will be used for cone-bioassays. In the field, where a rearing facility is unavailable, wild-caught blood-fed female mosquitoes are exposed to the surfaces for 30 minutes. After the exposure time, the mosquitoes should be carefully removed and placed in paper cups covered with nylon net fastened with a rubber band. Mosquitoes are to be provided with 10% glucose solution soaked in cotton wool and maintained in a climatic chamber or a room for 24 h maintained at 27°C ± 2°C and 80% ± 10% RH. After 24 h of holding, percent mortalities are computed from the total number of alive and dead mosquitoes (mosquitoes will be classified as dead if they are immobile or unable to stand or fly in a coordinated way) in the replicates for each type of surface and recorded in the data recording form. When the average mortality of the controls is between 5% and 20%, treated mortality will be corrected using Abbott's formula. But if control mortality is above 20%, the results will be discarded and the tests will be repeated. The inhabitants should be advised not to physically alter the marked areas and mud

Table 2.4: Details of spray coverage and insecticide consumption

Village .....PHC/CHC .....District .....State.....

Spray Squad No. ..... SFW/MTS/Supervisor..... Date of spray.....

Insecticide.....Formulation.....Dosage.....Spray round.....

Sl. No.	House No./HD or MD	Head of family	Sprayed	Locked	Refused	No. of rooms		*Room coverage (%)			Insecticide consumed (kg)
						Targeted	Sprayed	Houses	Cattle sheds	Temporary sheds	
01											
02											
03											
Total											

HD: Human dwelling; MD: Mixed dwelling; PHC: Primary Health Centre; CHC: Community Health Centre; SFW: Superior Field Worker; MTS: Malaria Technical Supervisor;

$$*\%\ \text{Room coverage} = \frac{\text{Number sprayed}}{\text{Number targeted}} \times 100$$

plaster/white wash/ paint the sprayed surfaces. After each exposure, the kit needs to be washed with soap and clean water and dried for next use. Results are expressed as residual activity (in number of weeks/ months) of the given insecticide(s)/ formulation(s).

The number of weeks/ months during which the mortality is above the cut-off level (at least 80% mortality after 24 h of holding) is recorded.

2.3.7. Evaluation

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

2.3.7.1. Efficacy

The expected effect of the insecticide used for IRS is to reduce the longevity, density and infectivity rate of the target vectors. Therefore, the following entomological parameters are relevant or required to assess the effectiveness of insecticide spraying.

1) Vector density [Relative abundance of vector(s) resting indoors and outdoors measured through hand catch (number per man-hour) or abundance indoors and outdoors measured using light traps (number per trap-night or number per trap-hour)].
2) Vector longevity (proportion parous)
3) Human landing density of vector(s)
4) Human blood index (a proportion that fed on human blood)
5) Entomological inoculation rate (product of man biting density and sporozoite rate)

Parameters 1–4 will be monitored in the randomly selected index villages. The methods of data collection/ processing of mosquito samples are described below:

2.3.7.1.1. 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 hour are one of the reliable and practical methods of assessing the population density of the vector species and also facilitates 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 collected mosquitoes are identified to species level 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 indicate 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 bloodmeal of individual mosquitoes is identified using precipitin (agar-gel diffusion method) or ELISA tests or PCR assays. Using the product of indoor resting collection and the proportion that fed on man (human blood index, HBI), the mosquito biting rate on humans may be estimated. Hand catches of indoor resting mosquitoes from six fixed (three human dwellings/houses and three cattle sheds) and six randomly selected catching stations (three human dwellings/houses and three cattle sheds) per village at fortnightly intervals will give meaningful data on mosquito density, which is expressed as the number per man-hour (Per man-hour density, PMHD). In case of the non-availability of cattle sheds, it should be compensated by selecting additional human dwellings.

Induced exophily/repellent effect: In addition, data on the exit rate (induced exophily) or repellent effect of the insecticide could be collected by fixing exit traps to the existing windows (if available and feasible) of the sprayed houses of the treatment and the positive control arms. Exit traps should be fixed at least in three houses in each of the six villages selected in each arm for indoor

resting collections. The traps should be fixed at dusk hour and removed at dawn hour and the female anophelines collected are identified to species and recorded arm-wise. In this 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 density: Outdoor resting mosquitoes could be collected from natural resting sites such as pit shelters, vegetation, root interstices and tree hollows available in and around the villages. However, searching for 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 from indoors by the excito-repellent effect of the insecticide. Nine to 12 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 at fortnightly intervals in the morning hours and the density is expressed as the number collected per shelter or man-hour. Collections and dissections of indoor and outdoor resting mosquitoes will be recorded in the format given in Table 2.5.

Mosquito landing density on human: The density of the vector species can also be monitored by conducting Human Landing Catches (HLC) that gives the number of landing mosquitoes per person per night. This may be done if feasible and on obtaining necessary clearance from the human ethics committee. All night (dusk to dawn) mosquito landing collections should be made in two to three villages in each arm (treatment and positive control), selecting one house in each village, at fortnightly intervals. Collections will be done indoors and outdoors, engaging human volunteers as baits to assess the landing rate on man indoors (endophagy) and outdoors (exophagy). Written consent from the human volunteers will be obtained in the information sheet, printed in a vernacular language containing all details of the study, before engaging as human bait for mosquito landing collections (Annexure 3). The human volunteers may lie down on a mat or a cot and can sleep as per their normal sleeping practice, exposing their legs up to their 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 2.6. Results are expressed as the 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.

The insect collectors who conduct human landing catches and the volunteers (baits) should be provided with malaria chemoprophylaxis and free medical care for up to three weeks after the end of the study.

Mosquito landing density 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 cow/buffalo tied to a pole are made from dusk to dawn. Data should be recorded in the format given in Table 2.7. Results are expressed as the number of vectors landing per animal bait per night or number of mosquitoes per bait per hour, if the collections are restricted to the hour of peak biting. This will provide information on the biting rhythm and feeding habits of the vector species in the area.

Table 2.5: Collection and dissection of mosquitoes resting indoors and outdoors

Sl. No.	House/ OD site No.	Man Hour Spent	Species	No. collected		No. dissected	Gonotrophic condition				Parity		Infection (Dissection)		Infection	
				♂	♀		UF	FF	SG	G	NP	P	Gland	Gut	+ve in ELISA	+ve in PCR
01																
02																
03																
04																
05																
06																

OD: outdoor; UF: Unfed; FF: Fully-fed; SG: Semi-gravid; G: Gravid; NP: Nulli-parous; P: Parous

Light trap catches: In areas with a significant positive correlation between the light trap (hung at the side of occupied untreated nets) catches and HLC, light trap catches can replace HLC. Mosquito collection using light traps is relatively easier and lesser labour-intensive than HLC using human baits. Therefore, light traps (CDC light traps or their modified versions) could be a reliable alternative that could overcome the ethical constraints and remove human error (while mosquito collection) associated with HLC. The traps are set indoors (human dwellings or animal sheds) near the bait and outdoors during dusk at fortnightly intervals in both treated and positive control villages, at least three villages in each arm. The next morning, the trapped mosquitoes are collected, identified to species and recorded in a format given in Table 2.8.

Table 2.6:. Mosquito landing collection on human (HLC)

Village.....Sub-Centre.....PHC/CHC.....District.....

State.....Insecticide/formulation.....Dosage Spray.....

Round..... Date of collection.....Temperature: Min.....Max.....

RH: Min..... Max.....

Time (Hrs)	Malaria vector (e.g. *An. culicifacies*)									Number collected					
	Number collected		Gonotrophic condition				No. dissected	Parity*		Other anophelines		*Cq*		Other culicines	
	♂	♀	UF	FF	SG	G		NP	P	♂	♀	♂	♀	♂	♀
18.00															
19.00															
20.00															
21.00															
22.00															
23.00															
24.00															
01.00															
02.00															
03.00															
04.00															
05.00															
06.00															

UF: Unfed; FF: Fully-fed; SG: Semi-gravid; G: Gravid; NP- Nulliparous; P- Parous; Cq: *Cx. quinquefasciatus*

*Unfed and freshly fed female mosquitoes should be dissected for parity, if tracheolar coiling is used and mosquitoes of all gonotrophic stages, if ovariolar dilatation method is followed.

Table 2.7: Mosquito landing collection on animal

Village.....Sub-Centre.....PHC/CHC.....District.....

State..... Insecticide/formulation.....Dosage.....

Spray..... Round..... Date of collection.....Temperature: Min.....

Max.....RH: Min..... Max.....

Time (Hrs)	Malaria vector (e.g., *An. culicifacies*)									Number collected					
	Number collected		Gonotrophic condition				No. dissected	Parity*		Other anophelines		*Cq*		Other culicines	
	♂	♀	UF	FF	SG	G		NP	P	♂	♀	♂	♀	♂	♀
18.00															
19.00															
20.00															
21.00															
22.00															
23.00															
24.00															
01.00															
02.00															
03.00															
04.00															
05.00															
06.00															

UF: Unfed; FF: Fully-fed; SG: Semi-gravid; G: Gravid; NP- Nulliparous; P- Parous; Cq: *Cx. quinquefasciatus*

*Unfed and freshly-fed female mosquitoes should be dissected for parity, if tracheolar coiling is used and mosquitoes of all gonotrophic stages, if ovariolar dilatation method is followed.

Table 2.8: Light trap collection

Village.....Sub-Centre.....PHC/CHC.....District.....

State..... Insecticide/formulation.....Dosage.....

Spray..... Round..... Date of collection.....Temperature: Min.....

Max.....RH: Min..... Max.....

Trap No.	Collection Site No. & Type	Malaria vector (e.g., *An. culicifacies*)									Number collected					
		Number collected		Gonotrophic condition				No. dissected	Parity*		Other anophelines		*Cq*		Other culicines	
		♂	♀	UF	FF	SG	G		NP	P	♂	♀	♂	♀	♂	♀
01																
02																
03																
Total																

UF: Unfed; FF: Fully-fed; SG: Semi-gravid; G: Gravid; NP- Nulliparous; P- Parous; Cq: *Cx. quinquefasciatus*

*Unfed and freshly-fed female mosquitoes should be dissected for parity, if tracheolar coiling is used and mosquitoes of all gonotrophic stages, if ovariolar dilatation method is followed.

2.3.7.1.2. Vector longevity

IRS is done primarily to reduce the longevity (survival) of vector mosquitoes and thereby interrupt the transmission of 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 or both. The ovaries of both unfed and 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. The dilatation method (Polovodova, 1949) can also determine the proportion parous. The mosquitoes having at least one dilatation in the pedicle part of their ovarioles are parous and those with none are nulliparous. If IRS with a given insecticide is effective, a marked reduction of parous mosquitoes in the population should be observed.

2.3.7.1.3. Infection and infectivity rates

Vector mosquitoes obtained from HLC, indoor and outdoor resting hand catches and pyrethrum spray 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 will survive the time required for sporozoites to develop and mature. 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 or PCR assay 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 make meaningful comparisons between study arms. Results are recorded in a format as given in Table 2.9. Data should be presented for each insecticide and dosage separately.

Table 2.9: Vector infection and infectivity rates

Village..... Sub-Centre..... PHC/CHC..... District.....

Date of collection ..... Method of collection..... Insecticide & Dosage.....

Dissected by..... Examined by..... ELISA done by.....PCR by.....

Species	No. dissected	Number +ve for		Oocyst rate	Sporo-zoite rate	No./No. of pools processed for ELISA	No. +ve in ELISA (%)	No./No. of pools processed for PCR	No. +ve in PCR (%)
		Oocyst	Sporo-zoite						
01									
02									
03									

$$\text{Oocyst/Sporozoite rate} = \frac{\text{Number found with oocysts/sporozoites}}{\text{Total number dissected}} \times 100$$

2.3.7.1.4. Entomological inoculation rate (EIR)

This is an important entomological indicator, which 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 disease transmission. EIR is an estimated number of infective bites received by a person per night through the vector population. It is the product of the sporozoite rate and the human landing density (number of mosquitoes landing per man per night). An effective insecticide treatment should reduce both the components of EIR.

$$EIR\ (h) = ma \times s$$

Where, ma = Man landing mosquito density (number landing per man per night) and

s = Sporozoite rate

2.3.7.1.5. Disease prevalence

Point prevalence of disease (malaria) in treatment and positive control areas should be assessed through sample blood surveys in each of the study arms. The sample size for blood survey should be estimated taking the immediate or average of the disease prevalence data available for the last three years in the study area. If prevalence data are not available, the data on slide/test positivity rate may be obtained from the NCVBDC, as a proxy and using this, the sample size can appropriately be estimated in consultation with a statistician. Minimum two sample blood surveys should be conducted, the first one 15 days prior to spraying (baseline) and the second one at the end of the study. Surveys may be carried out following a 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. Blood sample from a finger prick of the individuals in the selected households will be tested for malaria infection using rapid diagnostic kits (bivalent). Blood smears will be collected from RDT (bivalent)-negative patients and screened microscopically for malaria parasites other than *Plasmodium falciparum* and *P. vivax*. Written consent from them will be obtained in the information sheet, printed in a vernacular language containing all details of the study, before giving his/her blood sample (Annexure 4). In case of children, their assent/written assent should be obtained with the consent of their parents (Annexure 5 & 6). The test/ microscopic positive persons will be administered with anti-malaria drugs as per the NCVBDC guidelines. The health workers of the respective PHC/CHC will be involved in the treatment of malaria positive cases. Blood survey data are recorded in the format given in Table 2.10. Further, during analysis, the data may be arranged according to the following age groups; <1, 1–4, 5–8, 9–14 and ≥ 15 years (NCVBDC guidelines). The disease prevalence is expressed as slide/test positivity rate (SPR/ TPR):

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

2.3.7.1.6. Disease incidence

Fever surveillance should be carried out in the treated and the positive 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 (bivalent)-negative patients and screened microscopically for malaria parasites other than *Plasmodium falciparum* and *P. vivax*. Written consent from the fever patients will be obtained in the information sheet, printed in a vernacular language containing all details of the study, before giving his/her blood sample (Annexure 4). In case of children, their assent/written assent should be obtained with the consent of their parents (Annexure 5 & 6). All malaria positive cases will be administered with anti-malarial drugs following the NCVBDC Guidelines. Severe cases, if any, will be referred to the nearest PHC/CHC. The fever surveillance data will be recorded in the format given in Table 2.11. The incidence of malaria is expressed as incidence of cases against 1000 population on monthly (monthly parasite incidence, MPI) or yearly basis (annual parasite incidence, API). Data on malaria incidence may also be obtained from the State health facility.

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

Note: Owing to less endemicity in some areas, the data on disease prevalence need not be a factor to consider for deciding the efficacy of WHO PQ VC approved products/interventions. Epidemiological impact (on disease prevalence and incidence) should be assessed for new insecticide molecules/ formulations. Generating data on the reduction of prevalence/incidence is not mandatory for the insecticide molecules in use.

Table 2.10: Sample blood survey for disease prevalence

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

Date of collection.....Insecticide & Dosage.....Da te of spray.....Spray round.....
..

Survey done by..... Blood smears examined by..... Cross checked by.....

Sl. No.	House No.	Head of family	Name of patient	Age	Gender	Fever history/ Oral temp.	RDT result *Pf*/ *Pv*/ -ve	Blood smear No.	Smear examination result				Drugs given details	Remarks
									Pv	*Pf*	Mix	Others		
01														
02														
03														
04														
05														
06														

Table 2.11: Fever surveillance for disease incidence

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

Date of collection.....Insecticide & Dosage.....Date of spray.....Spray round.....

Survey done by..... Blood smears examined by..... Cross checked by.....

Sl. No.	House No.	Head of family	Name of patient	Age	Gender	Fever history: F,H,V, N,B,D	Oral temp	RDT result *Pf/ Pv/* -ve	Blood smear No.	Smear examination result *Pv/Pf/* Mixed/ Others	Parasite density	Drugs given	Remarks
01													
02													
03													
04													
05													
06													

F: Fever (C: Continuous), I: Intermittent, R: Remittent) H: Headache, V: Vomiting, N: Nausea, B: Body ache, D: Diarrhoea/ Dysentery, Pv: *P.vivax,* Pf: *P. falciparum,* -ve: Negative

2.3.7.1.7. Collection/survey methods and the parameters assessed

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

2.3.7.1.8. Adverse effects, acceptability by householders and collateral benefits

Acceptability of indoor residual spraying depends mainly on the benefits perceived by the population, the extent of inconvenience caused and any adverse side-effects produced by the insecticide spraying. Perceived menaces may lead to refusal among the households. Data on adverse effects, acceptability and collateral benefits are collected by interviewing the household head or an adult member of the family, who can give reliable information. Information will be collected from 30 households randomly selected within each treatment arm using a pre-tested structured questionnaire (Annexure 7) at the start of the intervention and every 6 months thereafter.

2.3.7.1.9. Human safety

Data on human safety will be collected by interviewing the spray-men and other handlers of the insecticide using a structured questionnaire (Annexure 8). A medical practitioner should be associated for collection of data.

2.3.7.1.10. Operational acceptability

In order to make the spraying operation as efficient as possible and to enhance ease of application, it is important to consider the following for understanding the operational acceptability of the insecticide or the insecticide formulation:

- % Suspensibility of the wettable powder formulation should be within the limits mentioned in the technical datasheet
- Difficulty in pumping, repeated clogging of nozzle, stability of suspension, maintenance need of equipment, nozzle corrosion, nozzle discharge rate etc. should be ascertained from spraymen and supervisory staff.
- Stability of insecticide suspension for a sufficient time after mixing
- Stability of insecticide formulations in different storage conditions
- Safety to spray-men and inhabitants (as investigated by using Annexure 8)
- Acceptability by community as determined by odour, effect on décor of premises, collateral benefits, etc. (as enquired by using Annexure 7)

2.3.7.1.11. 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 two key indicators. EIR, the product of sporozoite rate and mosquito landing density 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 the residual impact of the intervention over time (WHO, 2006).

3

Long-Lasting Insecticidal Nets (LLINs)

Use of insecticide treated nets (ITNs) is a preventative method for the control of malaria and kala-azar, as they prevent infective vector 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 minimizes consumption of insecticides in the control programme. Thus, the use of ITNs became an important component in malaria control, and many countries developed strategic plans to upscale the use. However, one of the operational challenges facing large scale implementation of ITNs programme was their re-treatment every six to 12 months with insecticides. Consequent to overall low re-treatment rates of ITNs, 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 conventionally treated ones.

The LLINs are nets of different fabrics such as high-density polyethylene (HDPE) or polyester. Insecticide treatment of LLINs is done at the factory level during the process of manufacturing. The net fibres are treated with insecticide following two techniques, (1) the insecticide is incorporated directly into the fibers 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. The bioavailability of the insecticide on the surface of the net will be sufficient to be lethal to vector mosquitoes for at least 3 years. Biological activity refers to the biological effect of LLINs on mosquitoes and as claimed by the manufacturer, it can be knockdown, deterrence, blood feeding inhibition, mortality, reduction in reproductive output or other impact on mosquito vectors that may reduce the transmission of vector-borne pathogens. The sponsor will provide candidate LNs (nets to be tested) and its specifications and appropriate reference nets (both positive {insecticide treated} and negative {untreated} control nets for comparison) for the trials.

New generation nets such as mixture nets (nets treated with a synergist and a pyrethroid on all panels) and combination nets (nets treated with a synergist and a pyrethroid on different panels, for e.g., Piperonyl butoxide, as a synergist, on roof panel and the synthetic pyrethroid on side

"Approved LLINs with particular specification and formulation are designated as LNs".
Example XXXXX LN, One thousand LLINs of XXXXX LN have been donated by the Company.

panels) are made available. These nets are claimed for management of pyrethroid resistant mosquitoes with major metabolic resistance mechanism involving monooxygenases.

Testing of LLINs involves laboratory evaluation (Phase I), small-scale field evaluation in experimental huts (Phase II) and large-scale field evaluation (Phase III). Laboratory evaluation estimates the regeneration time (time required to regenerate the insecticide on the net after 3 standard washes) and assess wash resistance (by estimating the bio-efficacy of nets against mosquitoes up to 20 standard washes) of the LLINs to guide experimental hut evaluation and to assist in the development of specifications of LLINs. The aim was not to simulate washing under field conditions. The key objective of these experiments is to provide a standardized uniform protocol which will allow reliable and consistent comparisons between laboratories and among different LLIN products. Small-scale field evaluation in experimental huts is conducted to establish the efficacy of LLINs against free flying natural mosquito populations. Large-scale field trials are designed to measure the duration in years the efficacy of LLINs lasts under user conditions i.e., for at least 3 years. Different parameters measured during different phases are as per the WHO guidelines (WHO, 2013) and are given below (Table 3.1).

Table 3.1: Different parameters measured during different phases

Phase	Objective	Parameters measured
Phase I: Laboratory evaluation	To determine efficacy of the product with active ingredient(s)	• Regeneration time for biological activity • Wash-resistance for biological activity • Chemical analysis for insecticide content
Phase II: Small-scale field evaluation (in experimental huts)	To determine efficacy of LNs in field against the target disease vector(s)	• Wash-resistance for biological activity • Efficacy indicators such as deterrence, blood-feeding inhibition, induced exophily and mortality. • Reduction in reproductive output (for some insecticides / compounds)
Phase III: Large-scale (village-scale) field evaluation	Bioefficacy and longevity of nets in field conditions	• Duration of biological efficacy • Chemical analysis for insecticide content • Physical integrity • Attrition rates • Community acceptance • Reported adverse events

3.1 Phase I: Laboratory evaluation

Duration: 12 months

Objective:

- To determine regeneration time for biological activity after washing
- To determine wash resistance for biological activity
- To analyze insecticide content of the nets after washes (chemical analysis)

3.1.1 Preparation of nets for testing

For Phase I study, four nets of the candidate LN from two batches are required. From each of the 4 nets, 14 pieces (25 cm x 25 cm) are sampled, as shown in Figure 3.1. Thus, a total of 56 pieces are sampled and used as follows:

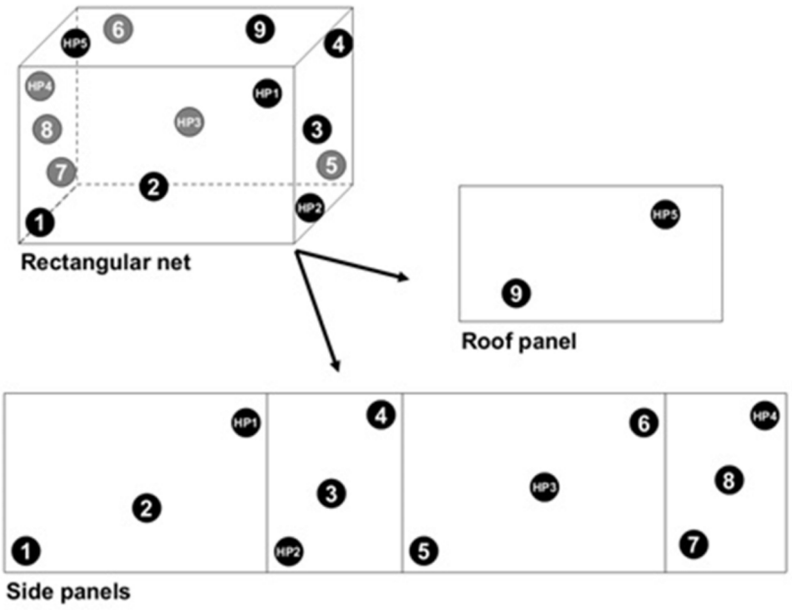

Figure 3.1: Sampling scheme for 14 pieces of netting from each net, including positions HP1–HP5 for chemical assay (Source: WHO, 2013)

Figure 3.2: Sampling and storage of nets pieces for the studies

Regeneration time: Eight (8) pieces (2 from each net) are used to estimate regeneration time with one piece designated as washed and the other unwashed, thus, 4 washed and 4 unwashed.

Wash resistance: Twenty-eight net pieces (7 from each net) are used to evaluate wash resistance. Four pieces are tested after 1, 3, 5, 10, 15 and 20 ($4 \times 6 = 24$ bioassays) washes. Twenty washes are considered standard for determining wash resistance. If the manufacturer claims more than 20 washes, additional net pieces may be cut and used for further washing and bioassays. After bioassays, the net pieces are tested in chemical assays to determine the wash-resistance index.

Chemical analysis: Twenty pieces (5 pieces from each of the 4 nets) are individually wrapped in aluminium foil and held at 4°C for chemical analysis to determine baseline chemical content, and variability of the A.I. between the pieces of a net and between the nets. This will be estimated following the CIPAC (MT/454/LN/M/3.2) protocol.

Nets pieces should be labelled and positioned to indicate the LLIN number and the position from which the pieces are cut (side 1–4 or Roof) as described in Figure 3.1. The net pieces are wrapped in aluminium foil and stored at 30°C between washes (Figure 3.2). Nets pieces are allocated randomly to each test procedure and should be handled with care to avoid contamination or excessive abrasion.

3.1.2 Washing of nets

Net samples designated for washing are introduced individually into 1-liter bottles (wide mouth glass bottle, eg., VWR Duran bottles) containing 0.5 l of double distilled or deionized water pre-heated to 30°C and containing 2 g/l soap (pH 10–11) added and fully dissolved. The bottles with

net pieces are placed securely in a shaker waterbath with warm water at 30°C and shaken for 10 minutes at 155 movements per minute. The samples are removed, rinsed twice for 10 min in clean, warm double distilled or deionized water under the same shaking conditions as above, and dried at room temperature. The procedure (washing) is repeated 3 times consecutively for regeneration time (Figure 3.3 & 3.4). Alternatively, soap (pH 10–11) can be prepared following the Collaborative International Pesticides Analytical Council (CIPAC) MT 195 (2014) and washed as per the CIPAC protocol. The washed net pieces are packed in aluminum foil, labelled and stored at 30°C in an incubator. These 3 times washed nets are subjected to cone bioassays by exposing mosquitoes that are susceptible to the insecticide in candidate LN on days 1, 2, 3, 5, 7 and longer, if necessary. The efficacy of nets is determined in standard WHO cone bioassays and/or tunnel tests depending on the claims of the manufacturer. All tests should be conducted at 27°C (±2°C) temperature and 75% relative humidity (±10%). Efficacy curves are established with this data in cone bioassays with days on X-axis and knock down/mortality on Y-axis.

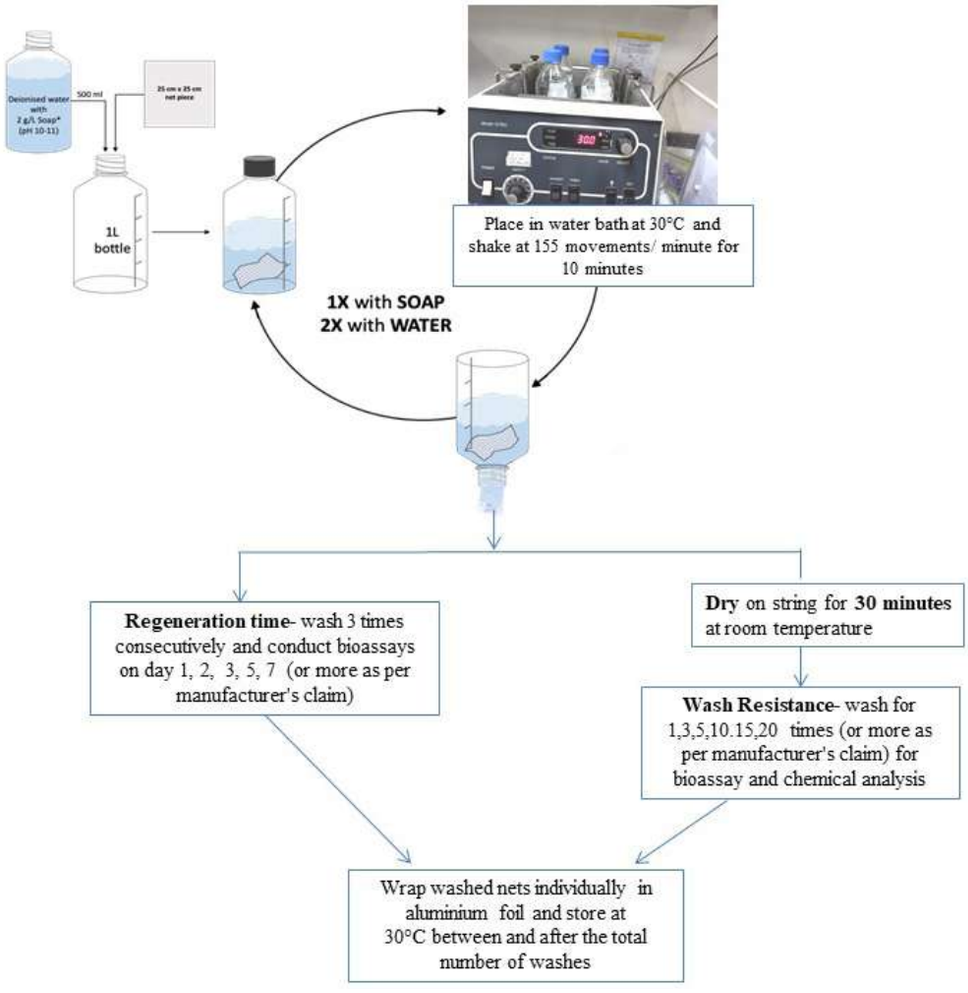

Figure 3.3: Schematic representation of washing procedure of nets pieces with soap for determining regeneration time and wash resistance

Figure 3.4: Washing of Net samples for regeneration time and wash resistance

3.1.3 Regeneration time

The 'regeneration time' is the time required to restore an effective insecticide concentration on the surface of the net after washing and is measured by bioassay. This information is important to determine the washing frequency and wash resistance at Phase II level. Baseline data on bio-efficacy is determined as knockdown (> 95%) or mortality (> 80%) on unwashed net pieces (25 cm x 25 cm) derived from the candidate LN using WHO cone bioassay.

3.1.3.1 Mosquito strains

Bioassays should be conducted with mosquito species that is susceptible to the insecticide in candidate LN. For the nets with claim for resistance management, resistant mosquito species should be used to justify the claim. The susceptibility of the mosquito colonies should be confirmed periodically preferably every 6 months by doing a susceptibility test (WHO, 2016).

3.1.3.2 WHO Cone-bioassay

Five non-blood-fed, 3–5-days-old female *Anopheles* (species and strain to be stated in the test report) mosquitoes are exposed to each piece of netting (25 cm x 25 cm) for 3 minutes in standard WHO cones (Figure 3.5). Cones and netting material should be held at 45° angle on the working bench. One net piece is to be tested randomly from 4 net pieces sampled from the four mosquito nets. Up to four cones at a time may be attached to a piece of netting, and five mosquitoes should be exposed in a cone first time. Second time, another five mosquitoes will be exposed to the same net piece, thus in total 10 mosquitoes will be exposed to a net piece. Thus, a total of 50 mosquitoes should be exposed to each net piece. Results should be reported for each net tested and for the four nets (4 pieces x 10 cone tests x 5 mosquitoes = 200 mosquitoes). Mosquitoes will be exposed to an untreated net piece in two cones as control (1 netpiece x 2 cones x 5 mosquitoes = 10 mosquitoes). They should be tested each day, with one control bioassay conducted at the beginning of a day of testing and a second bioassay conducted at the end of the day after bioassays on test nets have been completed to ensure that mortality is due to exposure to the LLIN and not due to handling of the mosquitoes. After 3 minutes exposure, mosquitoes are transferred to holding cups with access to 10% glucose solution in cotton swabs for knockdown and mortality assessment. All materials should be thoroughly cleaned each day to prevent contamination.

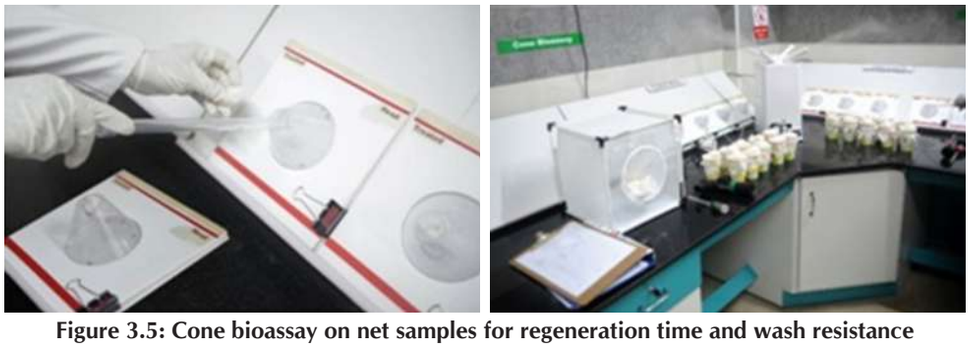

Figure 3.5: Cone bioassay on net samples for regeneration time and wash resistance

3.1.3.3 Determination of knockdown and mortality rates

Knockdown is measured after 60 minutes and mortality after 24 h holding period. The definitions of mortality and knockdown are those described by WHO (WHO, 2016). Mosquitoes are considered to be alive if they can both stand upright and fly in a coordinated manner. A mosquito is moribund if it cannot stand (e.g., has one or two legs), cannot fly in a coordinated manner or takes off briefly but falls immediately. A mosquito is dead if it is immobile, cannot stand or shows no sign of life. Mosquitoes that are moribund or dead are classified and recorded as knocked down at 60 minutes after the end of the exposure and as dead at 24 h or 48 h or observed beyond 48 h depending on the claims on period of bio-efficacy of the manufacturer. Mortality of mosquitoes exposed to test nets should be adjusted using Abbott's formula. If mortality in control replicates is >10% at 24 or 48 h, the results should be discarded. For mortality measured at 72 h, results should be observed till the cumulative mortality in the controls exceeds 20% (Table 3.2).

The time required (in days) to reach a plateau in efficacy is considered the regeneration time. If the manufacturer claims two or more effects of the insecticide such as knockdown, mortality, blood feeding inhibition, or other impacts such as reduction in reproductive output on mosquito vectors the efficacy curves are different.

Table 3.2: Cone bioassay

Lab/ F1/Field collected: ..... Test date..... ..... Test species: .....

Candidate LN: ..... Control LN: ..... Test done by.....

Supervised by.....

Replicates	Number knocked-down after 60 minutes	No. dead after 24 h	% Mortality	% Corrected mortality*	Remarks
Replicate 1					
Replicate 2					
Replicate 3					
Replicate n					
Control 1					

$$*\%\ \text{Corrected mortality} = \frac{(\%\ \text{Treated mortality} - \%\ \text{Control mortality})}{(100 - \%\ \text{Control mortality})} \times 100$$

3.1.4 Wash resistance

The resistance of an LLIN to washing is determined in standard bioassays with nets washed at intervals corresponding to the regeneration time (as determined above), using the standard wash procedure. Bioassays will be conducted prior to any wash ('0' wash) and after 0, 1, 3, 5, 10, 15 and 20 washes or more if claimed by the manufacturer (Figure 3.6). One piece of net is selected randomly from each of four nets. The efficacy of unwashed net piece is tested, but these pieces are not used for the chemical assay. The remaining pieces are randomly assigned and washed 1, 3, 5, 10, 15 and 20 times. After completing the assigned number of washes, the nets are tested for bio-efficacy through WHO cone bioassays (Refer to 3.1.1) and stored at 4°C for chemical analysis. All tests should be conducted at 27°C ±2°C and 75% ±10% relative humidity.

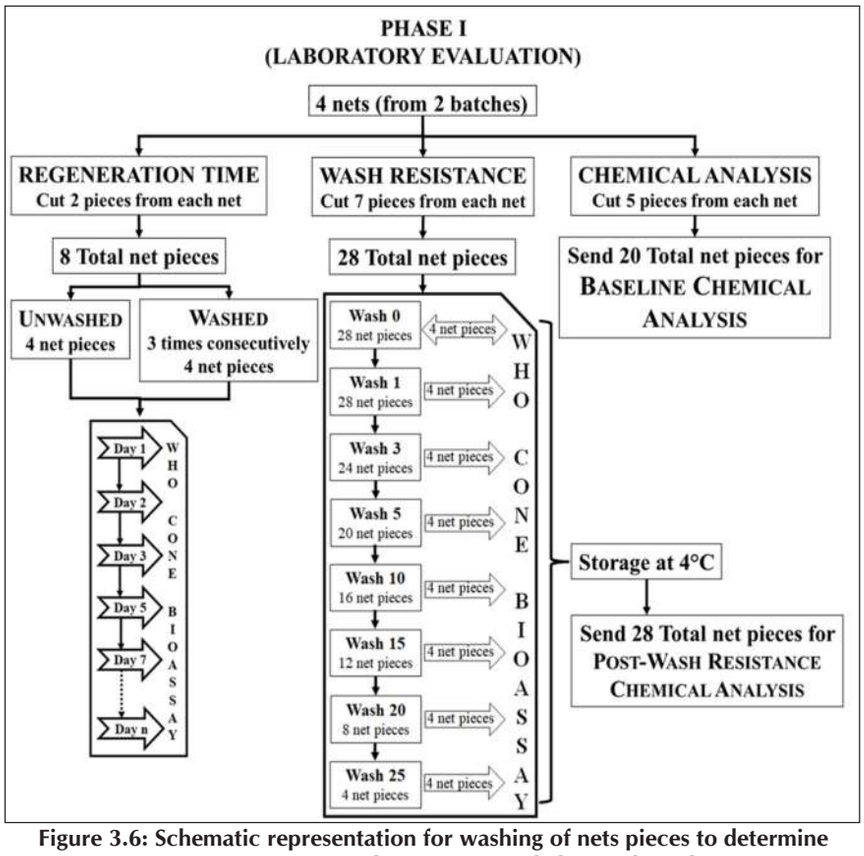

Figure 3.6: Schematic representation for washing of nets pieces to determine regeneration time, wash resistance and chemical residues

Each time, bioassay should be carried out just before the next wash. Percent knockdown and % mortality of mosquitoes or recorded against the number of washes, and the number of washes after which mortality or knockdown is above the cut-off level i.e., ≥80% mortality after 24 h or ≥95% knockdown 60 minutes after exposure, is also recorded. If the LN efficacy falls below the cut-off level, the evaluation should be continued to complete 20 washes and then a tunnel test is performed.

3.1.4.1 Assessment of blood-feeding inhibition and mortality

The nets that fail to meet the criterion of knockdown and mortality will be subjected to tunnel test to determine blood feeding inhibition. Some insecticides, such as permethrin and etofenprox, have a high excito-repellent effect, cause reduction in human-vector contact and biting. Although these insecticides also cause mortality, their main mechanism of action may be through irritancy or repellency, particularly as insecticide concentrations decline. In such cases or for novel insecticides with new modes of action that do not include direct mortality, the efficacy of LLINs washed 20 times or more should be studied in the tunnel test in the laboratory.

The tunnel test measures the mortality and blood-feeding success of host-seeking mosquitoes in an experimental chamber (Figure 3.7). The test is carried out in a laboratory by releasing 100 non-blood-fed female *Anopheles* mosquitoes aged 5–8 days and glucose starved for up to 12 h into a 60 cm tunnel (25 cm x 25 cm square section) made of glass. At each end of the tunnel, a 25 cm^2 cage covered with polyester netting is fitted (extension) (Figure 3.8). The LLIN sample, held in a disposable cardboard frame, is placed at one-third the length of the glass tunnel. The surface of netting available to the mosquitoes is 400 cm^2 (20 cm x 20 cm), with nine holes each 1 cm in diameter; one hole is located at the centre of the square, and the other eight are equidistant and located 5 cm from the border. In the shorter section of the tunnel (Figure 3.7, C2), a suitable bait (e.g., rabbit) of suitable size is placed by restraining it in a cage; the bait will be unable to move and available for mosquito biting. One hundred female mosquitoes are introduced into the cage at the end of the longer section of the tunnel (Figure 3.7, C1). They are free to fly in the tunnel but have to contact the piece of netting and locate the holes before passing through to reach the bait. After taking a blood meal, the mosquitoes may fly back to the cage at the end of this compartment and rest. Two replicate tunnels with candidate LN should be run. The experiment is set for 12–15 h (Day 1 - 6 pm to Day 2 - 6 am or 9 am). Two replicate tunnels with untreated netting should be used as a negative control for each night of testing.

During the tests, the tunnels and the cages are held at 27 ± 2°C and 75% ± 10% relative humidity at night in full darkness. After an exposure of 12–15 h, the mosquitoes are removed from each section of thetunnel with a glass suction tube and counted separately; mortality and blood-feeding rates are recorded. Blood-feeding inhibition is assessed by comparing the proportion of blood-fed females (alive or dead) in treated and control tunnels. Overall mortality is measured by pooling the mortality rates of mosquitoes from the two sections of the tunnel.

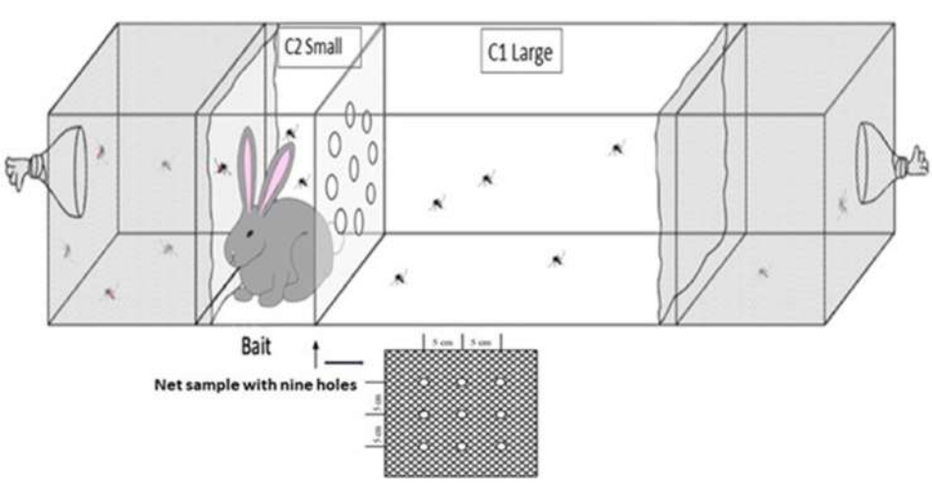

Figure 3.7: Graphic representation of tunnel test (courtesy: IHI, Tanzania)

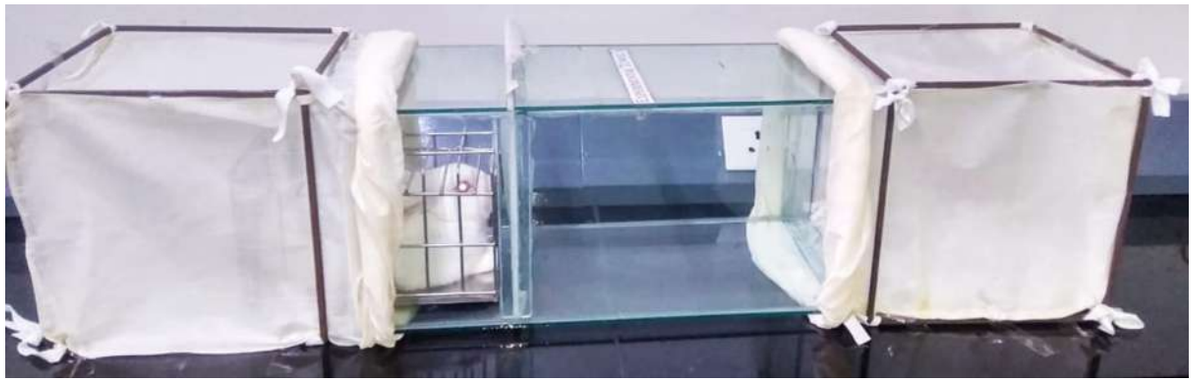

Figure 3.8: A complete set up of a tunnel with mosquito net, cage and rabbit (bait)

Mortality may be measured beyond 24 h up to a maximum of 72 h depending on the claims of the manufacturer. However, it is essential to monitor the mortality of mosquitoes exposed to an untreated control net to ensure that delayed mortality is due to exposure to the LLIN and not due to the handling of the mosquitoes. Mortality of mosquitoes exposed to test nets should be adjusted using Abbott's formula [% Corrected mortality ={(%Test mortality – % Control mortality)/ (100 – % Control mortality)} × 100]. For delayed mortality, results should be discarded if cumulative mortality in the controls exceeds 10% (Table 3.7).

The rate of blood-feeding by mosquitoes in the presence of insecticidal net samples has a considerable effect on mortality as the host-seeking behavior increases the chance of contact with treated nets. A 50% minimum cut-off value of the blood-feeding rate in controls should be established for tunnel tests.

Table 3.3: Mortality of mosquitoes exposed in tunnel test

Date of test:..... LN code: ..... Study code: .....

Temperature: °C ..... Relative humidity % ..... Test species and strain: .....

Age of mosquitoes..... Test start time* (h/min): ..... End date/ time (h/min)

..... Test performed by ..... Supervised by.....

Sample Type/ID	Tunnel Compartment	Blood fed Females		Unfed females		Total	
		Alive	Dead	Alive	Dead	Alive	Dead
Control	Small						
	Large						
Test 1	Small						
	Large						
Test 2	Small						
	Large						
Test 3	Small						
	Large						
Test 4	Small						
	Large						

3.1.4.2 Efficacy Criteria

For claims of efficacy, specific criteria should be met depending on the mode of action and one or more claim of the product such as knockdown, mortality, blood feeding inhibition, reduction in reproductive output or other impact on mosquito vectors. Ideally, as per the WHO definition, LN product should maintain stated efficacy for at least 20 washes or more as per the claim of the manufacturer.

Knockdown and mortality: For products that are claimed to cause knockdown or mortality of mosquitoes as measured in cone bioassays after 3 minutes exposure, there should be≥ 95% knockdown after 60 minutes of exposure or ≥80% mortality after 24 h holding. If the control mortality is ≤10% at 24 h, the test is considered valid.

Blood-feeding inhibition: For products that are claimed to cause reduced blood-feeding, mortality in the tunnel test should be ≥80% or blood-feeding inhibition should be ≥90%. Mortality of controls should be ≤10% and blood-feeding in the controls should be at least 50% for a test to be considered valid.

Efficacy Criteria for Phase I evaluation	Endpoints
Knockdown or Mortality	≥ 95% or ≥ 80%
Blood-feeding Inhibition	≥ 90%

3.1.5 Chemical analysis

Chemical analysis is not directly related to product claims but may be useful to understand the properties of the insecticide on the LLIN product and is necessary to support the development of product specifications for quality control. After cutting or testing for bioefficacy, all netting samples should be properly labelled, wrapped individually in aluminium foil and stored at 4°C until they are analyzed for their insecticide content to determine their wash-resistance index (w).

The wash resistance index (w) is determined as a percentage using the following formula:

$$w = 100 \times \sqrt[n]{(t_n / t_0)}$$

Where, n = number of washes, t_n = total active ingredient content (in g/kg) after n washing cycles; t_0 = total active ingredient content (in g/kg) before washing of nets (no washing).

The insecticide content of each net sample should be analyzed to estimate the average AI content as well as between- and within-net variation, and the fabric weight (i.e., mass of net per unit area) will also be measured. The net samples will be sent to GLP certified laboratories (https://ppqs.gov.in/sites/default/files/list_of_glp_nabl_accredited_laboratories_for_generating_data_on_registration_of_pesticides_as_on_01.10.2022.pdf) (Accessed on 15/12/2022) . The net samples should be analyzed following the method of Collaborative International Pesticides Analytical Council Limited (CIPAC) MT/454/LN/M/3.2. The results should be expressed in grams of active ingredient per kilogram fabric weight as well as in milligrams of active ingredient per square meter of netting material. Before washing, all LNs tested should comply with the manufacturer's specifications for the target dose (+25%). The decrease in insecticide content after successive washes can be used to estimate the wash-resistance index of the LN.

LNs washed at least 20 times that register a knockdown of ≥ 95% or mortality of ≥ 80% in WHO cone bioassays or ≥ 80% mortality or ≥ 90% blood feeding inhibition in tunnel tests meet the criteria to undergo phase II testing.

3.3 Phase II: Small-scale field evaluation (in experimental huts)

Duration: 18 months

Objectives

Small-scale field evaluation is carried out in experimental huts designed to simulate the local village huts. Overall, the purpose is to determine the efficacy of the candidate LN washed 20 times against free-flying, susceptible/resistant wild mosquitoes in comparison to a negative control (an untreated net with same netting material, mesh size and denier) and a positive control (a WHO PQT/VCP approved LN with same or similar specifications such as insecticide, method of impregnation, netting material, denier and mesh size) in experimental huts that match local domestic habitations. Additionally, impact of the insecticidal net on reproductive output can also be measured. Evaluation in experimental huts is to determine the efficacy of LLINs in field conditions, whereas, large scale field evaluation (at village level), carried out subsequent to Phase II trial, is to verify the claim for duration of efficacy of nets under field and user conditions.

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 verandah trap)
- 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 h holding alive mosquitoes, respectively).
- Impact on reproductive output (where applicable).

The above first three outcomes will be indicators of personal protection, and benefit individual users. The fact that blood-seeking females are killed will also be 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.

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

$$\% \text{ personal protection} = 100 \times (Bu - Bt)/Bu$$

Where, Bu = is the total number blood-fed in the huts with untreated nets, and Bt is the total number blood-fed in the huts with LN /treated nets.

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

$$\text{Mass killing effect (\%)} = 100 \times (Kt - Ku)/Tu$$

Where, Kt is the number killed in the huts with LN/treated nets, Ku is the number dying in the huts with untreated nets, and Tu is the total collected from the huts with untreated nets.

3.2.1 Characterization of vector population

In the study sites, the susceptibility of the wild population of the target vector species will be determined to the candidate insecticide (active ingredients in the net) at the beginning of the study and the population should be susceptible (Table 3.4). The susceptibility tests will be carried out following the WHO procedure (WHO, 2016). In the study sites with insecticide resistant vectors, the resistance level can be determined using 5 times and 10 times the discriminating concentration. This information would justify the appropriateness of using the available vector species in the test site as the target species to assess the efficacy of the candidate LN. For

example, if a candidate LN is treated only with a synthetic pyrethroid, it can be tested against a susceptible vector species, but if it is a PBO LN it would be appropriate to test against a resistant species. This would further help in interpreting the reliability of the results on the effectiveness of the candidate LN.

Table 3.4: Adult susceptibility test (Data capturing form for recording field observations)

Village:..... Sub-centre:.....

PHC/CHC: ..... District: ..... Ecotype: .....

Insecticide (%): ..... Test date:..... No. of times paper used: .....

Temp: Min: ..... Max: ..... Humidity: Min: ..... Max: .....

Impregnation date: ..... Expiry date:..... Test species:.....

Exposure time: .....minutes..... Lab./F1/Field collected:.....

Treatment Replicate	Number exposed[a]	Number knocked down in 1h	% Knock down	Number dead after 24 h	% mortality	Corrected mortality[b]
Treated replicates						
T1						
T2						
T3						
T4						
Total						
Control replicates						
C1						
C2						
Total						

[a]25 mosquitoes per replicate

[b] If the mortality in the control is $<5\%$, the test mortality is taken as such and no correction is required. If the control mortality is between $\geq 5\%$ and $\leq 20\%$, the test mortality needs to be corrected to the control mortality using Abbott's formula. When the mortality in the control is 20%, the test should be discarded and repeated.

$$\%\ \text{Corrected mortality} = \frac{(\%\ \text{Treated mortality} - \%\ \text{Control mortality})}{(100 - \%\ \text{Control mortality})} \times 100$$

Characterization of vector species for its insecticide susceptibility / resistance should also include determination of the resistance mechanisms (i.e., metabolic/target-site mediated resistance) and frequency. If the candidate LN is a pyrethroid-PBO net, presence of monooxygenases-based mechanism should be assessed, along with other reported mechanisms (WHO, 1998). Target site resistance genotyping alone is not currently acceptable for resistance profiling although it is a useful supporting evidence and to build up evidence base for their use in the future (Weill et al., 2004). In addition, in areas, where mosquitoes that would feed outdoors on cattle and enter huts to rest, a sample of mosquitoes from human-baited experimental huts should be identified to species and source of blood meals to determine the proportion of mosquitoes that fed on humans. This should be done preferably within a few weeks of the initiation of trial, as mosquito diversity / behavior may change over time due to the use of intervention.

3.2.2 Methodology

Phase II evaluation, after completing 20 washes and cone-bioassays on washed nets for bio-efficacy, will be continued in experimental huts constructed in village settings, where malaria

vector species (the target species) are more abundant. The number of huts required to be constructed/used will be based on the number of arms to be included for evaluation as per the trial design such as Non-inferiority or Superiority. Generally, the Phase II trial with non-inferiority trial design tests five arms viz., 1) Candidate LN, 2) Candidate LN washed 20 times, 3) Positive control LN (active comparator), 4) Positive control LN washed 20 times and 5) Negative control net (untreated net). Therefore, 5 huts are required to be constructed/employed, one hut for each arm. If it is a superiority trial design, one more arm i.e., a standard comparator (intervention/ product currently in use) will be added increasing the number of required huts to six. In other words, the number of huts will have to be decided as required by the agreed trial design.

3.2.2.1 Design of experimental huts

The experimental huts are specially designed for recording the entry and exit behavior of mosquitoes and for measuring their response to the insecticide(s) treated in the nets, including mortality. At the end of the study, the experimental huts can be renovated after sanitizing and validating the huts for decontamination from the previous insecticide exposures and used again. The experimental huts will have entry windows through which mosquitoes enter the huts and exit /veranda traps for measuring their exophily induced by the insecticide in the nets. Mortality (immediate and delayed) of the mosquitoes that entered the huts will also be recorded. The huts are surrounded by a water-filled moat to exclude ants and other scavengers.

3.2.2.2 Pre-hut trial assessment

Acclimatization: For acclimatization and to attract mosquitoes into the experimental huts, an adult volunteer enrolled for this purpose will sleep under an untreated mosquito net in each hut from dusk to dawn for 15 days. Clearance from the respective institute's ethics committee will be obtained to include human volunteers in the study.

Hut suitability: Subsequent to acclimatization, the suitability of the experimental huts for conducting Phase II trial will be assessed based on the following criteria over a period of one month prior to starting the hut-evaluation of the LNs.

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 (Table 3.5). Statistically comparable densities between the experimental and village huts indicate suitability of the experimental huts for evaluation.
2. **Tightness of huts (from recovery rate):** Around 75 (depending on availability) fully-fed field-collected female mosquitoes of the target vector species are released during one evening into each experimental hut and after the release the huts should be kept closed. The next day morning mosquitoes are recaptured. Recovery rate is the number of mosquitoes recaptured out of the total number released multiplied by 100. 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 anopheline mosquitoes are kept on the floor of the hut including verandah (in four corners) of each hut in the evening and the number present in the next day morning is recorded. Presence of all the dead mosquitoes indicates absence of scavenging and ensuring the suitability of the hut for trial. Such observations should be made on eight occasions, twice a week during the four weeks.

The experimental 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 (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 in the field.

3.2.2.3 Maintenance of the experimental huts

The rotation of the arms (nets) will be done every six days. On the 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.

3.2.3 Non-inferiority trial

The Phase II evaluation of LLINs in experimental huts will conventionally have a negative and a positive control. The negative control is an untreated net. All studies are required to include a negative control arm (an untreated net) to verify that the experimental hut trial is of sufficient quality to estimate natural mortality, blood-feeding and deterrence. Trials in which the overall 24-hour mortality in the control arm (over the length of the study) is >10 % need to be investigated and the trial needs to be repeated. Positive control will be a WHO PQT/VCP approved LN. Since, candidate products show significant variations in their design/formulation, it has become important to ensure a comparable performance of the candidate products to a first-in-class product (active comparator) so that there won't be any necessity for epidemiological impact assessment for each product. Therefore, the WHO has recommended a non-inferiority trial design for Phase II evaluation of new LNs using a defined set of entomological parameters (WHO/CDS/GMP/2018.22. Rev.1).

Non-inferiority trial margin

The aim of a non-inferiority trial is to assess that the test/candidate product is not inferior to the active comparator (positive control) by more than a small pre-determined margin (EMEA, 2005), which is called as the non-inferiority margin. For assessing non-inferiority of the test product, WHO has worked out an acceptable level of non-inferiority margin. In non-inferiority trials, new products are compared directly to the first-in-class product, which is referred to as the "active comparator". However, in case of difficulty in getting first-in-class product for comparison, a second-in-class product may be used as the active comparator provided it has been shown to be non-inferior to the first in class product based on the entomological parameters. If two or more products from the same insecticide class are available with sufficient epidemiological evidence for comparative evaluation, appropriate comparator can be selected by choice based on similar active ingredient (AI), design of the study or the mode of action etc. The non-inferiority margin has been defined as an odds ratio of 0.7 between the active comparator and the candidate LN. The odds ratio was selected for assessing non-inferiority, as the primary endpoints are dichotomous variables (i.e., a mosquito is either dead or alive, fed or unfed) and the odds ratio can be easily estimated using logistic regression. When the primary endpoint is the proportion of mosquitoes that are blood-fed, superior products should have lower values. In this case, the odds ratio is set at 1.43 (i.e., 1/0.7 = 1.43); a candidate product will show evidence of non-inferiority in terms of blood-feeding if the upper 95% CI estimate is lower than 1.43. An odds ratio of 0.7 equates to a difference in percentage mortality of no more than 9%.

Primary endpoints

Two primary endpoints should be considered relative to the first-in-class product

- mosquito mortality and
- mosquito blood feeding inhibition

Secondary endpoints

- Deterrence (95% CI) - The reduction in the number of mosquitoes entering an experimental hut with an intervention relative to a control hut.
- Induced exophily (95% CI) - The proportion of female mosquitoes in exit traps and verandah compared to total females in the hut

- Additional primary and secondary end-points will be identified for novel class of LNs based on the manufacturer's claim on bio-efficacy e.g., Insecticide-insecticide/insecticide-synergist/ insect growth regulator etc. as to the increased holding time (48, 72 h)/ reproductive outputs (reduction in fecundity, hatchability, larval survival and adult emergence).

Statistical analysis

i. To relate the outcome variables to the intervention and covariates, generalized linear regression models (GLMs) should be used. For binary endpoints, such as the proportion of mosquitoes dying or feeding, a logistic model is appropriate.

ii. For outcomes that are counts, such as the number of mosquitoes entered, a Poisson or negative binomial model may be more appropriate. The models should include huts, sleepers and number of washes for LLINs as fixed or random effects. The intervention itself should be included as a fixed effect. The estimated effect of the intervention and 95% CIs should be reported in all instances.

Interpretation of Non-inferiority test

The candidate product is deemed non-inferior if:

a. The lower 95% CI estimate of the odds ratio describing the difference in mosquito mortality between the candidate and the active comparator product is greater than 0.7.

b. The upper 95% CI estimate of the odds ratio describing the difference in mosquito blood-feeding between the candidate and active comparator product is greater than 1.43.

3.2.4 Superiority trial

In case, a new product is claimed to be superior over the currently used product in the control programme (an intervention belonging to another/older class), then it is necessary to demonstrate that the new/candidate product is superior, in terms of efficacy, over the current standard of care intervention in use in the field for vector control (*standard comparator) based on entomological end points (WHO, 2019).

If a candidate or a new LN product is not shown to be better than the current standard of care, it is still essential to prove that it is non-inferior to the standard of care (which will be the active comparator) and superior to the negative control arm (untreated net) of the trial (this is to prevent candidate second-in-class products from being evaluated in sites where first-in-class products are no longer fully effective).

The candidate product is classified as superior if:

a. A significantly higher proportion of mosquitoes have died at a 5% significant level ($p < 0.05$) in comparison to the negative control or standard comparator.

b. It has a significantly lower proportion of mosquitoes that have blood fed at 5% significance level ($p < 0.05$) when compared to the standard comparator or the negative control.

3.2.5 Organization of trial

Prior to evaluation of nets in the experimental huts, washing of the nets will be carried out for 20 times using non-detergent soap (pH 9–10). The duration of net washing will be 2–5 months depending on the regeneration time as determined in phase I. Acclimatization of experimental huts and assessment of their suitability may take about 2 months.

Hut evaluation will be done over a minimum period of 6 weeks (6 weeks x 6 days) or 12 weeks or more (it should be a multiplication of 6 weeks) depending on the availability of the vector mosquitoes in adequate number.

In addition, post-evaluation activities will include chemical analysis of net pieces and data analysis and report writing. The initiation of the trial will be decided in such a way that the

hut evaluation period should coincide with the season of vector abundance so as to ensure adequate sampling.

3.2.5.1 Experimental Arms

Unwashed and 20 times washed candidate LNs will be evaluated in experimental huts for their efficacy against free-flying, natural population of the vector species and for their ability to deter entry, repel or drive mosquitoes out of houses, inhibit blood-feeding and induce mortality.

The allocation of arms will be made as per the need of the trial design, non-inferiority or superiority. Generally, the Phase II trial with non-inferiority trial design tests five arms as given below:

Arm 1: Unwashed candidate LN

Arm 2: 20 times washed candidate LN

Arm 3: Unwashed positive control (active comparator) LN

Arm 4: 20 times washed positive control (active comparator) LN

Arm 5: Untreated net (negative control)

Therefore, 5 huts are required to be used, one hut for each arm. If it is a superiority trial design, two more arms, i.e., unwashed standard comparator (LN currently in use) and 20 times washed standard comparator, will be added to the above five arms, thus increasing the number of required huts to seven.

Untreated nets of the same material will be used as a negative control. WHO PQT/VCP approved LN (with the same or similar specifications) washed 0 and 20 times will be the positive controls. Normally, the nets will be of the following size: approx. 205–220 cm long, 170–175 cm wide, 150–155 cm high. The nets (candidate LN, active comparator LN, negative control net and standard comparator, if applicable) will be provided by the sponsor.

3.2.5.2 Preparation of nets

The nets will be coded (Codes X1, X2, X3, X4, X5, X6 and X7 to indicate the seven arms (or up to X5 if the trial has only five arms) and letter code A, B, C, D, E, F and G to indicate the seven replicate nets of each arm, and Y and Z to indicate the additional nets of the arms, Y- before any wash, Z- 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.

Seven 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 and X1G in which X1 will be the experiment arm and A, B, C, D, E, F and G will be its replicates).

3.2.5.3 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 / resistant vector mosquitoes (as per the claim of the product).

Cone-bioassay before any wash and after washing 20 times will be conducted. Also, bioassay using non-blood fed susceptible/resistant vector species will be done on one net per arm just before the experimental hut evaluation (one randomly selected net out of the seven replicate nets, A, B, C, D, E, F and G of each arm). At the end of the experimental hut trial, bioassay will be performed on one used net per arm (one randomly selected net out of the seven replicate nets used in the huts).

For cone bioassay, 5 x 2 cone tests will be performed per net (on each section of the net: roof and 4 sides) (as shown in figure 3.9). Five female mosquitoes will be exposed per cone (Table 3.6)

Table 3.5: Comparison of density of target vector species between experimental and village huts (for assessment of hut suitability)

Place of collection:.....Name of study site: Village.....District:.....Date of collection: .....

..... Man-hour spent in each hut: .....

Expt. Hut No.	No. of anophelines collected		No. of mosquitoes of the target vector species collected		PMD (♂)		Village Hut No.	No. of anophelines collected		No. of mosquitoes of the target vector species collected		PMD (♀)	
	♂	♀	♂	♀	Anophelines	Target vector species		♂	♀	♂	♀	Anophelines	Target vector species
Total/Average													

Table 3.6. Results of cone bioassay on nets

Test Species: ..... Lab/ F1 /Field collected: .....Date of Test:.....

Arm code: ..... Replicate code: ..... Test done by .....

Supervised by.....

Arm	Number exposed	Number knocked down after 60 minutes	No. dead after 24 h	% Mortality	% Corrected mortality*	Remarks

$$*\%\ \text{Corrected mortality} = \frac{(\%\ \text{Treated mortality} - \%\ \text{Control mortality})}{(100 - \%\ \text{Control mortality})} \times 100$$

Exposure of mosquitoes on net will be for 3 minutes and after which the mosquitoes are held for 24 h with access to cotton pads soaked in 10% glucose solution. Knockdown (KD) is recorded 60 min after the exposure time and mortality after 24 h. Results are pooled for the 50 mosquitoes tested per net (5 net pieces from each net, 10 mosquitoes on each net piece i.e., 5 mosquitoes 1st time and 5 mosquitoes 2nd time).

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.

3.2.5.4. Chemical analysis

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-X7Y) of each of the seven arms (Figure 3.9 - cutting of nets). Similarly, net samples will be obtained from the additional net (X1- X7Z) after 20 washes for insecticide content analysis. At the end of the experimental hut study, one used net of each arm will be sampled in the same way as described above. The samples will be labelled and packed in aluminium foil and stored at 4–8°C until sending them for insecticide content analysis to a GLP certified lab following CIPAC or any other WHO approved method. The insecticide content of each net sample will be analyzed (net pieces cut from position number 1 will be analyzed separately) (Section 3.1.2) to estimate the average AI content as well as between- and within-net variation. Before washing, all LNs tested should comply with the manufacturer's specifications for the target dose (±25%). The results should be expressed in both grams of active ingredient per kilogram and milligrams of active ingredient per square metre of netting. The decrease in insecticide content after successive washes can be used to estimate the wash-resistance index of the LN (WHO/HTM/NTD/WHOPES/2013.1).

3.2.5.5 Procedures for tests in huts

Tests will be done in seven or required number of experimental huts; in each hut one net will be used (Table 3.7 & 3.8). Holes will be made in all nets of the experimental arms (seven replicate 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 on long sides and one each on front and hind end (Figure 3.10).

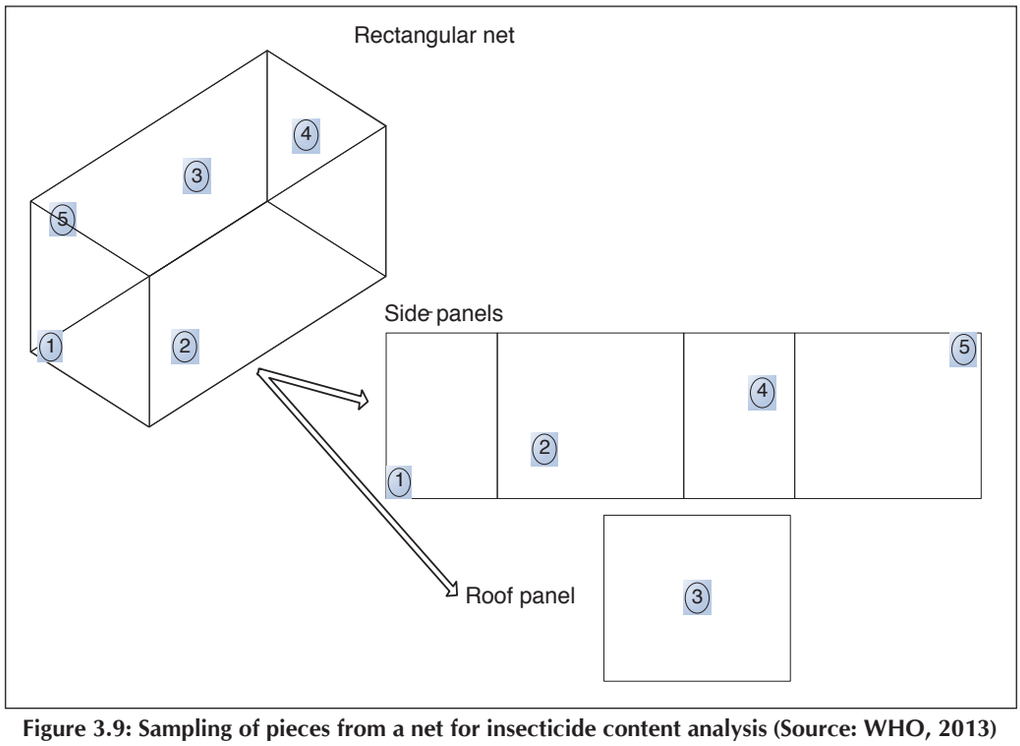

Figure 3.9: Sampling of pieces from a net for insecticide content analysis (Source: WHO, 2013)

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

To sleep in the experimental huts, depending up on the number of huts to be used, number of volunteers (e.g., 14 volunteers for seven huts @ two per hut mostly husband and wife), 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 decided time, at 18.00 /19.00 h (dusk) and remain inside until 05.30 h (dawn). Inside the hut, they will sleep under the net assigned to that hut. Each volunteer will be compensated for wages as per the local government and with the approval of human ethics committee.

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.

3.2.5.7 Rotation of treatments and volunteers

The treatment arms will be rotated among the huts each week according to the Latin square or any other appropriate rotation scheme (Table 3.7 & 3.8), 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 9) and necessary medical care will be provided free of cost.

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 labeled 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 (labeled 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 rotation should be long enough to obtain sufficient number of mosquitoes for adequate statistical analysis.

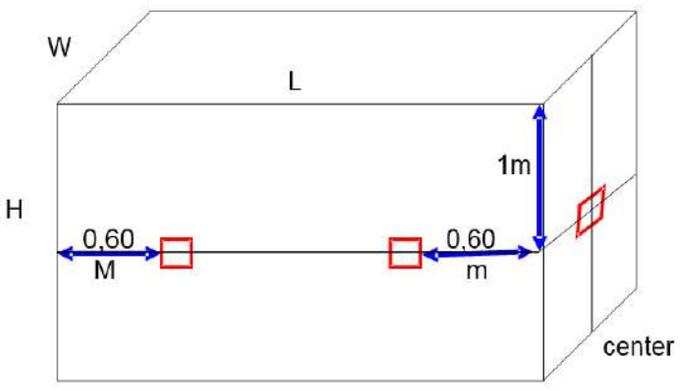

Figure 3.10: Location of the holes on net

Table 3.7: Latin Square rotation scheme for seven experiment arms (nets and huts) and sleepers

Rotation of experiments arms										Rotation of teams						
Week	Day	H1	H2	H3	H4	H5	H6	H7		H1	H2	H3	H4	H5	H6	H7
1	1	X1	X2	X3	X4	X5	X6	X7		A	B	C	D	E	F	G
	2	X1	X2	X3	X4	X5	X6	X7		B	C	D	E	F	G	A
	3	X1	X2	X3	X4	X5	X6	X7		C	D	E	F	G	A	B
	4	X1	X2	X3	X4	X5	X6	X7		D	E	F	G	A	B	C
	5	X1	X2	X3	X4	X5	X6	X7		E	F	G	A	B	C	D
	6	X1	X2	X3	X4	X5	X6	X7		F	G	A	B	C	D	E
	7	X1	X2	X3	X4	X5	X6	X7		G	A	B	C	D	E	F
	8	Ventilating, cleansing, washing of hut								No volunteers sleeping inside the hut						
2	9	X2	X3	X4	X5	X6	X7	X1		A	B	C	D	E	F	G
	10	X2	X3	X4	X5	X6	X7	X1		B	C	D	E	F	G	A
	11	X2	X3	X4	X5	X6	X7	X1		C	D	E	F	G	A	B
	12	X2	X3	X4	X5	X6	X7	X1		D	E	F	G	A	B	C
	13	X2	X3	X4	X5	X6	X7	X1		E	F	G	A	B	C	D
	14	X2	X3	X4	X5	X6	X7	X1		F	G	A	B	C	D	E
	15	X2	X3	X4	X5	X6	X7	X1		G	A	B	C	D	E	F
	16	Ventilating, cleansing, washing of hut								No volunteers sleeping inside the hut						
3	17	X3	X4	X5	X6	X7	X1	X2		A	B	C	D	E	F	G
	18	X3	X4	X5	X6	X7	X1	X2		B	C	D	E	F	G	A
	19	X3	X4	X5	X6	X7	X1	X2		C	D	E	F	G	A	B
	20	X3	X4	X5	X6	X7	X1	X2		D	E	F	G	A	B	C
	21	X3	X4	X5	X6	X7	X1	X2		E	F	G	A	B	C	D
	22	X3	X4	X5	X6	X7	X1	X2		F	G	A	B	C	D	E
	23	X3	X4	X5	X6	X7	X1	X2		G	A	B	C	D	E	F

	24	Ventilating, cleansing, washing of hut							No volunteers sleeping inside the hut						
4	25	X4	X5	X6	X7	X1	X2	X3	A	B	C	D	E	F	G
	26	X4	X5	X6	X7	X1	X2	X3	B	C	D	E	F	G	A
	27	X4	X5	X6	X7	X1	X2	X3	C	D	E	F	G	A	B
	28	X4	X5	X6	X7	X1	X2	X3	D	E	F	G	A	B	C
	29	X4	X5	X6	X7	X1	X2	X3	E	F	G	A	B	C	D
	30	X4	X5	X6	X7	X1	X2	X3	F	G	A	B	C	D	E
	31	X4	X5	X6	X7	X1	X2	X3	G	A	B	C	D	E	F
	32	Ventilating, cleansing and washing of hut							No volunteers sleeping inside the hut						
5	33	X5	X6	X7	X1	X2	X3	X4	A	B	C	D	E	F	G
	34	X5	X6	X7	X1	X2	X3	X4	B	C	D	E	F	G	A
	35	X5	X6	X7	X1	X2	X3	X4	C	D	E	F	G	A	B
	36	X5	X6	X7	X1	X2	X3	X4	D	E	F	G	A	B	C
	37	X5	X6	X7	X1	X2	X3	X4	E	F	G	A	B	C	D
	38	X5	X6	X7	X1	X2	X3	X4	F	G	A	B	C	D	E
	39	X5	X6	X7	X1	X2	X3	X4	G	A	B	C	D	E	F
	40	Ventilating, cleansing and washing of hut							No volunteers sleeping inside the hut						
6	41	X6	X7	X1	X2	X3	X4	X5	A	B	C	D	E	F	G
	42	X6	X7	X1	X2	X3	X4	X5	B	C	D	E	F	G	A
	43	X6	X7	X1	X2	X3	X4	X5	C	D	E	F	G	A	B
	44	X6	X7	X1	X2	X3	X4	X5	D	E	F	G	A	B	C
	45	X6	X7	X1	X2	X3	X4	X5	E	F	G	A	B	C	D
	46	X6	X7	X1	X2	X3	X4	X5	F	G	A	B	C	D	E
	47	X6	X7	X1	X2	X3	X4	X5	G	A	B	C	D	E	F
	48	Ventilating, cleansing and washing of hut							No volunteers sleeping inside the hut						
7	49	X7	X1	X2	X3	X4	X5	X6	A	B	C	D	E	F	G
	50	X7	X1	X2	X3	X4	X5	X6	B	C	D	E	F	G	A
	51	X7	X1	X2	X3	X4	X5	X6	C	D	E	F	G	A	B
	52	X7	X1	X2	X3	X4	X5	X6	D	E	F	G	A	B	C
	53	X7	X1	X2	X3	X4	X5	X6	E	F	G	A	B	C	D
	54	X7	X1	X2	X3	X4	X5	X6	F	G	A	B	C	D	E
	55	X7	X1	X2	X3	X4	X5	X6	G	A	B	C	D	E	F
	56	Ventilating, cleansing and washing of hut							No volunteers sleeping inside the hut						

NETS:	SLEEPERS:
ARM X1, nets A,B,C,D,E,F,G	A: Team A – Two Volunteers
ARM X2 nets A,B,C,D,E,F,G	B: Team B – Two Volunteers
ARM X3 nets A,B,C,D,E,F,G	C: Team C – Two Volunteers
ARM X4 nets A,B,C,D,E,F,G	D: Team D – Two Volunteers
ARM X5 nets A,B,C,D,E,F,G	E: Team E – Two Volunteers
ARM X6 nets A,B,C,D,E,F,G	F: Team F – Two Volunteers
ARM X7 nets A,B,C,D,E,F,G	G: Team G – Two Volunteers

Table 3.8: Latin Square ROTATION scheme for experiment arms, nets* and sleepers (5 arms)

		Rotation of experiments arms						Rotation of teams				
Week	Day	H1	H2	H3	H4	H5		H1	H2	H3	H4	H5
1	1.	X1	X2	X3	X4	X5		A	B	C	D	E
	2.	X1	X2	X3	X4	X5		B	C	D	E	A
	3.	X1	X2	X3	X4	X5		C	D	E	A	B
	4.	X1	X2	X3	X4	X5		D	E	A	B	C
	5.	X1	X2	X3	X4	X5		E	A	B	C	D
	6.	Ventilating, cleansing and washing of hut						No volunteers sleeping inside the hut				
2	7.	X2	X3	X4	X5	X1		A	B	C	D	E
	8.	X2	X3	X4	X5	X1		B	C	D	E	A
	9.	X2	X3	X4	X5	X1		C	D	E	A	B
	10.	X2	X3	X4	X5	X1		D	E	A	B	C
	11.	X2	X3	X4	X5	X1		E	A	B	C	D
	12.	Ventilating, cleansing and washing of hut						No volunteers sleeping inside the hut				
3	13.	X3	X4	X5	X1	X2		A	B	C	D	E
	14.	X3	X4	X5	X1	X2		B	C	D	E	A
	15.	X3	X4	X5	X1	X2		C	D	E	A	B
	16.	X3	X4	X5	X1	X2		D	E	A	B	C
	17.	X3	X4	X5	X1	X2		E	A	B	C	D
	18.	Ventilating, cleansing and washing of hut						No volunteers sleeping inside the hut				
4	19.	X4	X5	X1	X2	X3		A	B	C	D	E
	20.	X4	X5	X1	X2	X3		B	C	D	E	A
	21.	X4	X5	X1	X2	X3		C	D	E	A	B
	22.	X4	X5	X1	X2	X3		D	E	A	B	C
	23.	X4	X5	X1	X2	X3		E	A	B	C	D
	24.	Ventilating, cleansing and washing of hut						No volunteers sleeping inside the hut				
5	25.	X5	X1	X2	X3	X4		A	B	C	D	E
	26.	X5	X1	X2	X3	X4		B	C	D	E	A
	27.	X5	X1	X2	X3	X4		C	D	E	A	B
	28.	X5	X1	X2	X3	X4		D	E	A	B	C
	29.	X5	X1	X2	X3	X4		E	A	B	C	D
	30.	Ventilating, cleansing and washing of hut						No volunteers sleeping inside the hut				

NETS*	SLEEPERS
ArmX1,nets A,B,C,D,E	A : Team A - two volunteers
ArmX2,nets A,B,C,D,E	B : Team B - two volunteers
ArmX3,nets A,B,C,D,E	C : Team C - two volunteers
ArmX4,nets A,B,C,D,E	D : Team D - two volunteers
ArmX5,nets A,B,C,D,E	E : Team E - two volunteers

* Each net will be used only one night per week, see labeling in Annexure 1.

3.2.5.8 Collection and processing of mosquitoes

In the morning mosquito collections will be made from 05.30 hour separately from inside the net, veranda and the hut.

Dead mosquitoes will be picked up from the floor of the hut and inside the nets. Resting mosquitoes will be collected using aspirators from inside the net and from the walls and roof of the hut and verandah trap. Mosquitoes will be stored separately by hut and by collection place (veranda trap, room, inside bed net) for further processing. Mosquitoes found on the net (on the outside) will be classified as in the "room/hut".

All mosquitoes will be processed as follows:

a) The collected mosquitoes will be identified morphologically. The anophelines will be identified using the standard key and *Culex* spp. will be grouped together.

b) Mosquitoes will be scored as dead or alive

c) Physiological status will be scored (blood fed/unfed/ semi-gravid + gravid)

d) Alive mosquitoes will be placed by species, collection place and physiological status in cups with access to glucose solution for 24 h. Humidity and temperature will be controlled during this period, and access to ants will be prevented. After 24 h, delayed mortality will be recorded.

e) Results will be recorded on the daily record sheet

f) One form will be completed per hut, per species and per week (one form for each target vector species, one for other anophelines and one for culicines)

3.2.6 Efficacy criteria

For claims of efficacy, specific criteria should be met depending on the mode of action claimed by a product (insecticide used in the net) as outlined below and the number of washes after which a product meets one or more of the criteria may be listed as part of the product claims.

Knockdown or mortality

For products that claim to cause mortality of mosquitoes as measured in cone bioassays, there should be ≥95% knockdown after 1 h of exposure or ≥80% mortality after 24 h holding period or for extended holding periods up to 72 h (or more as claimed by manufacturer/sponsor) after exposure, but the period to reach 80% mortality need to be considered. If the mortality in controls <10% at 24 h or for extended periods the test is considered valid. If the mortality in control replicates is >10% the test to be discarded. For extended holding periods the control mortality can be <20%.

Blood-feeding inhibition

Blood feeding inhibition rate is estimated from the number of blood fed mosquitoes out of the total mosquitoes entered the hut. The inhibition rates obtained for the candidate LN will be compared with the positive (active/standard comparator) and negative (untreated net) controls.

Reduction in reproductive output

For products with claims of reducing reproductive output, fecundity, hatchability, larval survival and adult emergence will be calculated for individual arms from the mosquitoes that entered the huts and found fed and alive, will be compared between the different arms for any reduction with the candidate LN

3.2.7 Perceived adverse/beneficial effects

The volunteers sleeping in the huts should be enquired about any perceived adverse or beneficial effects of the LNs. Volunteers reporting any adverse events associated with use of treated nets will be given medical care, if necessary. Such observations can provide initial evidence of the types and frequency of adverse events associated with a product. However, experimental hut studies are not designed to evaluate the safety of LN products in the field as the small number of participants and their rotation among treatment arms preclude any association between a given LN product and adverse events.

3.2.8 Ethical considerations

The experimental hut studies involve risk, as volunteers are asked to sleep under nets in which holes have deliberately been made. Furthermore, they are asked to sleep one night per week under nets that are untreated. Although the sleepers will probably be at lower risk than if they had not used a net, the investigators should minimize the risk. Only adults (excluding pregnant women) should be allowed to participate, and, depending on the setting, it may be advisable to allow only adult males to participate. Participants should be given chemoprophylaxis as per the national guidelines. National ethical guidelines should be followed for the study. The experimental hut evaluation involving human volunteers should be approved by the respective institute's/organization's human ethics committee. Insurance cover of the volunteers should be provisioned in the budget as approved by ethics committee of the respective institution.

An information sheet must be given or read to all sleepers participating in experimental hut studies, apprising them of the procedures involved. Written informed consent must be obtained. A generic consent form for sleepers is shown in Annexure 1. It is the responsibility of the principal investigators to ensure that necessary clearances or waivers are obtained before starting experimental hut study. A statement that the study was approved by the concerned human ethics committee should be included in any reports submitted for review.

3.3 Phase III: Large-scale (village-scale) field evaluation

Duration: Three years (additional 3 months for preparation, data analysis and report writing)

Large-scale field trials are conducted at least in three eco-epidemiological settings. Phase III trial will demonstrate community acceptability, fabric integrity and insecticidal activity of nets during the 3 years of field use.

Objectives

- To evaluate the insecticidal activity and fabric integrity of the candidate LN over 36 months in comparison to a WHO PQT/VCP approved LN in the same field conditions.
- To assess LLIN washing mode and washing habits of the householders.
- To assess the side/adverse effects perceived by the community and community acceptability of LN.

A candidate LN is considered to meet the phase II efficacy criterion if the net, after 20 washes, performs equal to or better than the reference LN in terms of blood feeding inhibition and mortality.

3.3.1 Methodology

The phase III field trial is a prospective study in which the candidate LN is evaluated in a two-arm randomized trial against a WHO PQT/VCP approved reference LN (positive control) of similar specifications in terms of insecticide, impregnation technology, netting material, denier and mesh size.

Insecticidal efficacy, fabric integrity and washing practices of the householders and their perception on side/adverse effects, if any, will be assessed during the study. The efficacy of candidate LN in comparison to the positive control LN distributed in the villages will be monitored up to three years of continuous use under the field conditions.

3.3.1.1 Selection of villages

The phase III evaluation will be carried out in villages selected on the basis of population size, malaria incidence, accessibility and community use of nets in consultation with the State/District Health Authorities. The selected villages will be randomly assigned to either of the two arms (candidate LN or reference LN). A total of 460 nets of the candidate LN and 460 nets of the positive control LN (reference LN) will be distributed to the households of the villages as assigned for destructive sampling to assess bioefficacy and chemical content. The nets will be distributed to the households at the rate of 1 net for 2 persons. In addition, for each arm, another 250 nets will be distributed to a cohort of 250 households (one net per household) for attrition survey and among the 250 nets, 150 nets will be randomly selected and surveyed for fabric integrity.

3.3.1.2 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 gender of every family member will be recorded in registers**.** A baseline household survey will be carried out in all the selected villages using a structured questionnaire. Respondents will be heads of households or their spouses or any other adult representatives. 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 (to estimate the net requirement), presence of mosquito nets/ LLINs in households, their usage pattern, washing practices etc.

The data recorded in community registers and questionnaires in the field will be transferred into a computer data file (the householders will be impressed upon to use the LLINs provided in the study and avoid using other available nets in the household).

3.3.1.3 Community education and informed consent procedure

As the trials include community based activities, the assistance of community leaders, elected leaders (President of Village/ Panchayat samiti) and local health personnel (PHC staff, ASHAs, ANMs, malaria workers) in the selected villages will be sought (i) to obtain permission to use the community as a study site, (ii) to inform the community members about the purpose of the study, consequent sampling procedures and replacement of sampled net with new ones and (3) to seek community acceptance for use. In addition, community level meetings will be organized to inform all people in the selected villages on the adverse consequences of malaria, benefits of using long-lasting insecticidal nets, correct handling and use of nets and the need for reporting adverse events, if any, and to seek their support and cooperation 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. A draft consent form is attached vide (Annexure 9). 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 witness from the community. Upon their consent, the 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 (Annexure 10). 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 for medical attention. They will be advised to report all such events to the research team. The team will also facilitate the treatment, if required.

3.3.1.4 Withdrawal of participants

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.

3.3.1.5 Distribution of LLINs

Each net of the two arms will be allotted an ID. The ID numbers will be written with wash-resistant ink on a piece of polyester band 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. Required number of nets (if necessary uncoded nets) will be provided to each household to ensure complete coverage. The nets will be given free of charge to all households and acknowledgments will be received for the receipts of nets.

At the time of distribution of LLINs, every headperson of the household will be informed about the appropriate use and maintenance of net. The need for reporting adverse effects, if any, during/after using the nets will be emphasized. 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. The research team and local health personnel will repeatedly emphasize on the net use.

The following steps may be taken to encourage proper use of trial nets and coverage by study participants:

- Use of old nets should be discouraged for use by householders or participants should be encouraged to use only the trial nets for the duration of the study
- Nets should not be distributed in their original sealed package.
- Before distribution, nets should be coded (as described above) and saved in a secondary paper or polythene pack.
- Field teams should assist to hang the nets by providing materials such as hooks, ropes or poles etc.
- Householders should be asked to start using their nets from the day of distribution.
- 'Hang-up' campaign may be conducted by visits of the teams to households to ensure that recipients are using their new nets. Households that are not using their nets should be revisited.

3.3.2 Sampling of LLINs

3.3.2.1 Chemical assays

Samples of LLINs will be analyzed for chemical content in a GLP certified lab following Collaborative International Pesticide Analytical Council (CIPAC) or any other WHO approved method. To ensure that the target dose of the insecticide on the net has been achieved, netting pieces will be cut at the beginning of the trial for baseline assays. Thirty nets at baseline should be used for chemical analysis with five net piece samples from each net (5 x 30 = 150 pieces at baseline, positions HP1-5 of each net). Sample cutting should be according to Figure 11. Thereafter, sampling should be done at 12-, 24-, and 36-months post-distribution. Net pieces will be cut for 30 nets at each time point (positions 2-5).

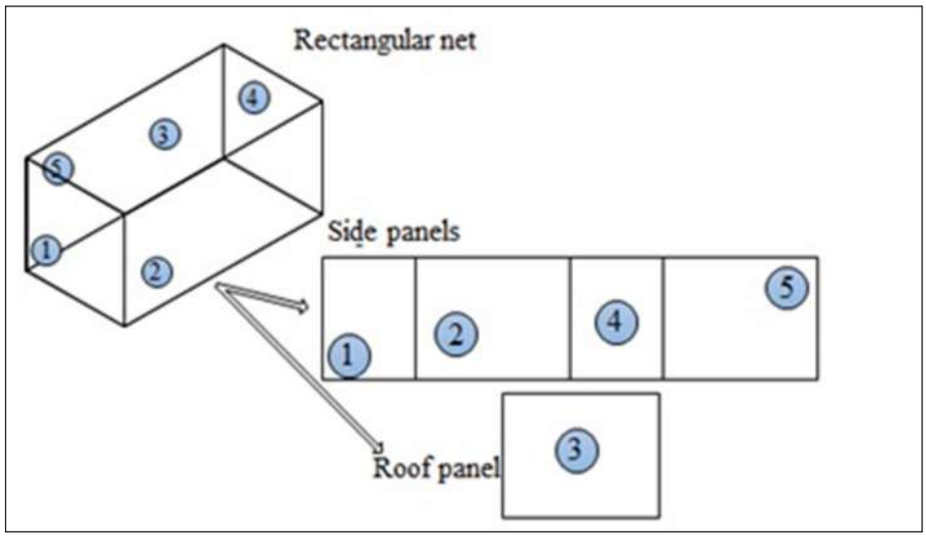

Figure 3.11: Positions on nets for drawing netting pieces

Thus, from each of the 30 sampled nets of each arm, four pieces of 30 cm x 30 cm size will be cut from positions 2, 3, 4 and 5 using sharp scissors*. The sub-samples will be rolled up and placed in new, clean and labelled aluminium foil for storage at 4 to 8°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 AI content of the insecticide in the LLINs under evaluation. Simultaneously, net pieces adjacent to the place from where net pieces were cut for chemical content analysis, will be cut (25 cm x 25 cm) and used for cone bioassays.

3.3.2.2 Insecticide susceptibility of target vector species

Female mosquitoes of the target vector species will be collected from the study sites using mouth /mechanical aspirators and flashlight and maintained in the laboratory to lay eggs that will be used for rearing F1 progeny for susceptibility tests. The laboratory reared 3 to 5 days old, non-blood-fed female mosquitoes will be exposed to the discriminating concentration of the insecticide used in the candidate LN to determine resistance/susceptible frequency using WHO adult susceptibility kits (WHO 2016) (Table 3.9).

3.3.2.3 Bio-efficacy of LNs through cone-bioassays

The standard WHO procedure (cone bioassay) will be used for evaluation of insecticidal efficacy of LNs (WHO, 2005). Accordingly, at the start of the trial and at every 6 months thereafter, 30 nets of each arm (candidate or positive control LN) will be randomly drawn from the net master-list by the principal investigator and used by the research team for collection of net 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 from positions 1 to 5 as shown in Figure 3.11 and used for the bioassays. Pieces will be cut as squares using sharp scissors. Bioassays will be done using cones on all the five pieces. Five laboratory-reared 3 to 5 days old; non-blood-fed F1 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 on each of the five netting samples in

**For 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).*

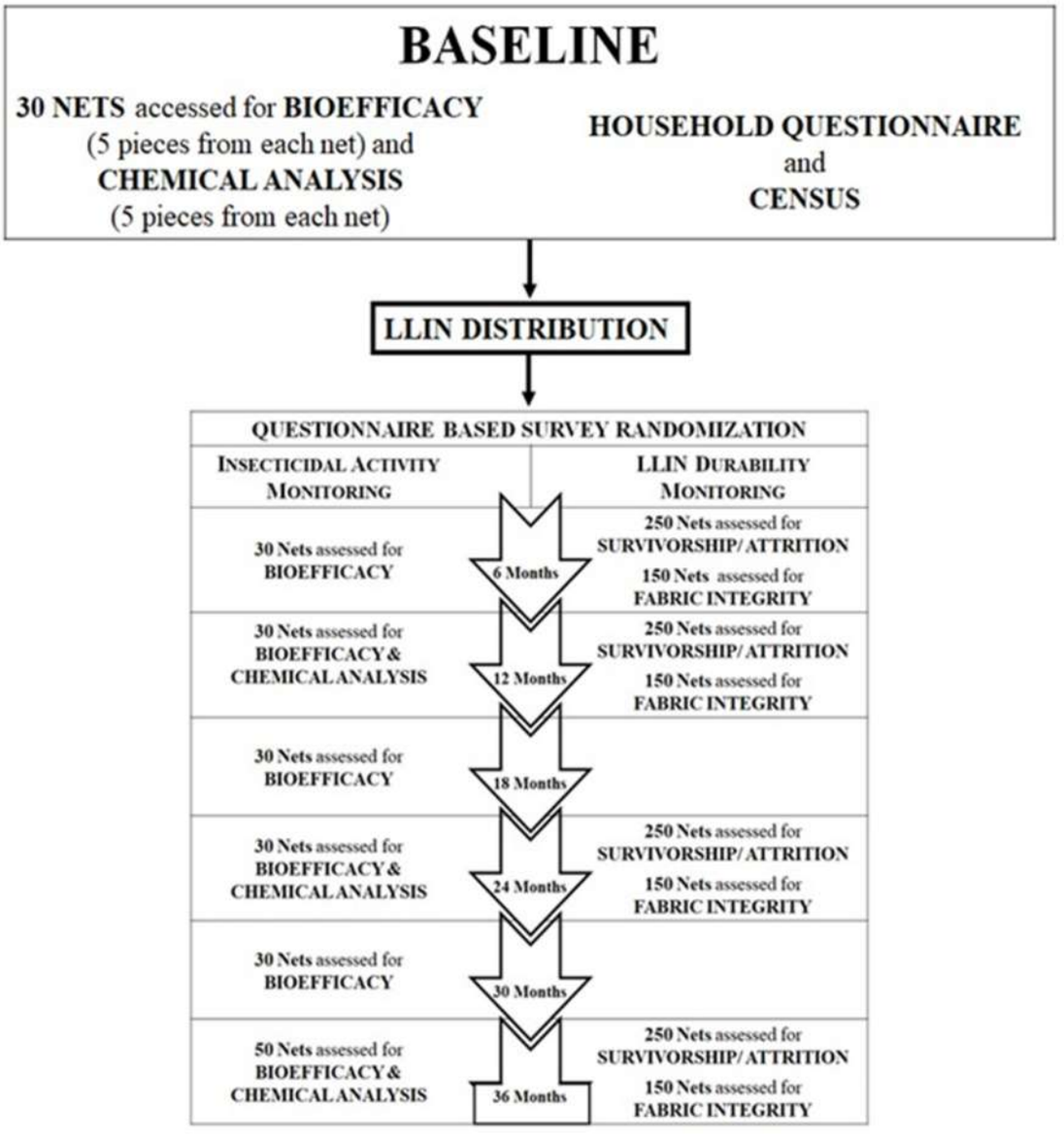

Figure 3.12: Schematic representation for washing of nets pieces to determine regeneration time, wash resistance and chemical residues

baseline (positions 1–5) and four netting samples (positions 2–5, position 1 is excluded because of the possible abrasion during use) of nets collected at 0, 6, 12, 18, 24, 30 and 36 months. Cone bioassays will be done on 4 net pieces of each net by exposing 10 mosquitoes (5 mosquitoes × 2) and 2 control replicates. Two control replicates (2 cones × 5 mosquitoes on untreated net pieces) are exposed each time and for extended testing additional 2 control exposures will be made.

After the exposure, the mosquitoes will be removed gently from the cones and kept separately in labelled paper cups provided with cotton-wool soaked in 10% glucose solution on the nylon netting fastened to the rim. Knockdown will be recorded after 60 minutes and mortality after 24 h holding. Mosquitoes exposed to untreated nets will be used as controls. The bioassays will be done and the mosquitoes will be held at 27 ± 2 °C and 75 ± 10% RH. Data will be recorded in the prescribed format, as given in Table 3.10, and analyzed.

The bioassays will be done once at the start of the study as explained above, and at every 6 months thereafter up to 3 years (Figure 3.12). Nets selected randomly and withdrawn from a household for destructive sampling will be replaced with a new LLIN. If there are significant variations in bioassay results, mean results of positions 2 to 5 will be used.

Table 3.9: Insecticide susceptibility test (WHO tube method)

Village..... Sub-centre.....PHC/CHC.....District .....

Insecticide (%).....Impregnation date ..... Expiry date:.....

No. of times paper used.....Date of Test.....Temp: Min..... Max .....

Humidity: Min ..... Max ..... Test species .....

Lab/F1/Field collected..... Exposure: Start time ..... End time.....

Holding: Start time..... End time.....

Replicate	No. exposed*	No. knocked down in 1 h	No. dead after 24 h	% Mortality	% Corrected mortality#	Remarks
Treated 1						
Treated 2						
Treated 3						
Treated 4						
Treated total						
Control 1						
Control 2						
Control total						

*25 mosquitoes per replicate; #After using Abbott's formula when mortality in control replicates is between ≥5 and ≤20% (If control mortality is <5% no correction is needed and > 20% test to be discarded).

$$\%\ \text{Corrected mortality} = \frac{(\%\ \text{Treated mortality} - \%\ \text{Control mortality})}{(100 - \%\ \text{Control mortality})} \times 100$$

Test done by..... Supervised by.....

Table 3.10: Cone bioassay on nets

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

Candidate LN ..... Reference LN ..... Exposure time.....

Test done by..... Supervised by.....

Net Piece#	Number knocked-down after 60 minutes	No. dead after 24 h	% Mortality	% Corrected mortality@
Net piece 1				
Net piece 2				
Net piece 3				
Net piece 4				
Net piece 5				
Control 1*				
Control 2*				

Candidate LN/Reference LN
* Untreated net piece

$$@\%\ \text{Corrected mortality} = \frac{(\%\ \text{Treated mortality} - \%\ \text{Control mortality})}{(100 - \%\ \text{Control mortality})} \times 100$$

3.3.2.4 Tunnel test

LLINs which caused a knockdown of <95% and a mortality of <80% in cone bioassays 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, starved up to 12 h in a tunnel (square section 25 cm x 25 cm) made of glass, 60 cm length (WHO, 2013) (for details of carrying out tunnel test, please refer to section 3.1.4.1).

3.3.3 Sample size for assessing different attributes of LLINs in field use

Community acceptance and assessment of adverse effects

A team of staff will be trained on administering the questionnaire and conduct the survey.

An assessment of adverse effects, if any, among candidate / reference net users will be made using a questionnaire as given in Annexure 10 during the periodic surveys. The Principal Investigator will select 30 ID numbers from the candidate LN group and another 30 from the positive control LN group from the respective master list using a random selection procedure. The list of selected nets will be re-sorted in ascending order and given to the field team visiting 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.

At the end of month(s) 1, 6, 18 and 30, an adult member in each of the 30 selected households will be interviewed by door-to-door visit to assess net utilization pattern/frequency of use (including early morning observations), mode of washing and number of washes and type of detergent used and physical integrity of the net (size and number of holes) as per the questionnaire (Annexure 11). Since interview assessment of washing frequency may not always be reliable, the nets should be looked for the presence of the marking with the water-soluble ink.

At the end of months 6, 12, 24 and 36, surveys will be conducted by visiting door-to-door to record attrition of nets (physical presence/absence) and fabric integrity. To estimate the attrition rate and to collect information on people's perceptions and practices as mentioned above including any adverse effects observed, questionnaires given in Annexure 10 and 11 will be used for the surveys.

Attrition rate will be calculated by observing a minimum of 250 nets per arm. It is assumed that if there are initially selected 250 LNs in each arm for attrition survey, at least 150 LNs per arm will remain available for inspection at the end of the 3-year study. A list of randomly selected nets with their unique code numbers and information on the household to which they were distributed (e.g., household identification, name of head of household, GPS coordinates) should be given to the staff who are sampling the nets in the field.

Fabric integrity will be measured by observing a sub-sample of 150 nets of the 250 nets distributed for assessment of attrition.

Monitoring attrition and fabric integrity

During surveys of LLIN attrition and fabric integrity, a standard questionnaire should be used to collect data on the status (community acceptability, physical integrity and mode of washing of nets) of each LLIN and on their use and handling (Annexure 11).

The use of mobile technology (e.g., personal digital assistants, tablets or smartphones equipped with GPS) for recording responses to the questionnaire is recommended for automated data checking. GPS readings, scanning bar codes on nets if provided by the manufacturer and photographic records of nets in the field are also valuable.

Sampling of nets for insecticidal activity and chemical content analysis

Nets should be sampled for insecticidal activity at 0, 6, 12, 18, 24, 30 and 36 months after distribution to determine their bio-efficacy. Net pieces should be cut from each site as indicated in Figure 3.11. Adjacent netting pieces are cut for chemical assays from the nets sampled for bioassays at 0, 12, 24 and 36 months. All pieces should be labelled to indicate the specific LLIN

and location from which they were cut. Thirty nets are sampled at each timepoint except for 36 months when 50 nets should be sampled Figure 3.12.

A sub sample of nets assigned to the cohort for bio-efficacy testing should be randomly selected for destructive sampling and replaced by new LLINs of same brand. As nets may be lost to follow-up, it is best to anticipate the number of losses and randomly select more than 30 LLIN codes from the master list, so that at least 30 nets can be identified at the time of sampling. It has also been found useful to update the master list of nets after each follow-up survey to eliminate the codes of lost nets and re-randomize for sampling just before the next follow-up survey (Table 3.11).

Table 3.11: Sample calculation and sample size required for each arm of the study at each follow up for measuring attrition, fabric integrity and testing bio-efficacy and chemical content

Period of sampling	Cohort of nets to be inspected				Number of nets to be withdrawn for bio-efficacy and for chemical analysis	
	Candidate LN		Positive control LN			
	Number of nets for attrition[a]	Number of nets for fabric integrity[b]	Number of nets for attrition[a]	Number of nets for fabric integrity[b]	Candidate LN	Positive control LN
At week 1 for baseline bio-efficacy (cone bio-assay) & chemical assay	-	-	-		30[c]	30[c]
At 6 months for bio-efficacy	250	150 (sub-sample of 250)	250	150 (sub-sample of 250)	30[d]	30[d]
At 12 months for bio-efficacy and chemical assay	250	150	250	150	30[c]	30[c]
At 18 months for bio-efficacy	-	-	-	-	30[d]	30[d]
At 24 months for bio-efficacy and chemical assay	250	150	250	150	30[c]	30[c]
At 30 months for bio-efficacy	-	-	-	-	30[d]	30[d]
At 36 months for bio-efficacy and chemical assay	250	150	250	150	50[c]	50[c]
Total number of nets distributed	**250**		**250**		**460**	**460**

Source: WHO, 2013

Note that the table provides sample sizes for two different groups of nets under study. The first group will be followed-up for net attrition and field estimates of fabric integrity. These nets will not be withdrawn. The second group will be followed-up for bio-efficacy and chemical analysis and will require withdrawal of nets at each follow up.

[a] For net attrition in a 3-year study, a minimum of 250 nets will be distributed. The same nets will be followed-up in each survey as long as they will be available with the households included in the study cohort. When nets will be no longer present, the reason for their loss would be recorded.

[b]Field measurement of fabric integrity will be done on a sub-sample (150 nets) of the nets (250 nets) distributed for assessment of attrition. Note that the sample size for fabric integrity represents a minimum target. Where possible, it would be ideal to measure fabric integrity on all nets that will be followed for attrition. If sampling will be done at household level, the number of households sampled should be adjusted to reflect the estimated number of nets per household.

[c]Cone bioassay (for bio-efficacy) and chemical content analysis will be done on 30 nets drawn randomly from the coded nets available in the households at different intervals as specified in the Table

[d] Only cone bioassay (for bio-efficacy) will be done on 30 nets drawn randomly from the coded nets available in the households at different intervals as mentioned in the Table.

3.3.4 Outcome measures

1.1.3.1 Net attrition

To measure survivorship or attrition, the physical presence of the LLIN in the household should be recorded during each follow-up survey. The investigator should record whether the net is being used for its intended purpose. Nets that have never been used or are used for other purposes should be recorded as present but should be excluded from the analysis. If the net is no longer in the house, the investigator should ask the owner the reason for missing (Annexure 11).

Attrition should be determined for all nets recorded during the exercise at each interval but stratified by LLIN product. The number of nets in the sample, the proportion of the indicator and the 95% confidence interval should be reported (taking account of the sampling design, i.e., cluster sampling, if applicable). The following indicators should be used and disaggregated by survey time (e.g., 6, 12, 24 or 36 months, or more if necessary):

1.1.3.2 Attrition rates (survivorship of nets in households)

The numerator is the total number of LLINs of each arm (candidate or positive control/reference LN) present in the surveyed households (and available for sleeping under) × 100. The denominator is the total number of LLINs of each arm distributed to the surveyed households in the study cohort at the beginning of the study.

Survivorship rate = (LLIN present during the survey /LLINs Distributed) × 100

Attrition is calculated as 1 minus survivorship. Attrition can be due to discarding of nets because of excessive loss of fabric integrity (true attrition); movement of nets for sale, giving them away or using them in another location (migration); or use for other than the intended purpose, although still owned by the household (misuse). Nets that are worn out but stored in the house and no longer used for their original purpose should be considered to have undergone true attrition. Attrition due to migration or misuse is likely to occur with any type of net, whereas true attrition may be associated with the physical characteristics of the net. The cause of true attrition can be further disaggregated according to the type of damage, such as wear and tear from regular use or damage due to animals or fire.

For each product, the non-response rate or the proportion of nets that cannot be traced should also be reported, as high non-response rates may indicate a bias.

3.3.4.3 Fabric integrity: (Source: WHO, 2013)

Fabric integrity is assessed from the questionnaire by counting the number of holes (including tears and split seams) by their location on the net and their size. Holes can be classified into:

size 1: smaller than a thumb (0.5–2 cm),

size 2: larger than a thumb but smaller than a fist (2–10 cm),

size 3: larger than a fist but smaller than a head (10–25 cm) and

size 4: larger than a head (> 25 cm).

Holes smaller than 0.5 cm should be ignored. Evidence of repairs to the net fabric and the type of repair should also be recorded on the form.

In follow-up field surveys, holes in nets are usually counted in the field. Nets should be examined outside, either held by at least two people and inspected by a third or draped over a portable frame. In some cases, the cause of the holes may be deduced from their physical characteristics or by questioning the user (Annexure 11).

Note: Numbers of nets for testing is the minimum number. Actual number of nets distributed depends on attrition (net loss) in an area of distribution.

The three indicators of interest are the proportion of LNs with holes, the hole area and the hole index.

For the proportion of LNs with any holes (with 95% confidence interval), the numerator is the total number of each LN product with at least one hole of size 1–4, while the denominator is the total number of each LN product found and assessed in surveyed households. This indicator may also be calculated for each category of hole size.

The hole area is calculated by assuming that the holes in each size category are circular, with a diameter that is equal to the mid-point of the category (except for the largest category, for which an arbitrary diameter (30 cm) is selected, as there is no upper limit). For the four sizes listed above, the diameters would be 1.25 cm, 6 cm, 17.5 cm and 30 cm, respectively. The area (A) of each hole can then be estimated from the equation:

$$A = \pi r^2,$$

where $\pi = 3.142$ and r = the diameter divided by 2 and summed over each net (Table 3.12). For the hole categories listed above, the estimated hole areas are 1.23 cm^2, 28.27 cm^2, 240.53 cm^2 and 706.85 cm^2.

Table 3.12: Calculation of hole index

Hole size (cm)	Hole diameter (d; cm)	Hole radius ($r = d/2$)	r^2	Area of hole (πr^2)	Hole Index[a] (pHI)
0.5 – 2.0	1.25	0.625	0.39063	1.23	1
2–10	6	3	9.0	28.27	23
10–25	17.5	8.75	76.5625	240.53	196
>25	30[b]	15	225.0	706.85	576

[a]area divided by 1.23; [b]assumed diameter

The hole index is calculated by weighting each hole by size and summing for each net. If the weight of hole sizes 1, 2, 3 and 4 is A, B, C and D, respectively, the hole index is calculated as:

Hole index = (A x no. of size-1 holes) + (B x no. of size-2 holes) + (C x no. of size-3 holes) + (D x no. size-4 holes).

For each product type, the mean (and standard deviation) as well as the median (and interquartile range) hole index should be determined. The hole index for different products can be compared by analysis of variance for normally distributed data or the Kruskal-Wallis test for data that is not normally distributed. The data on fabric integrity is important and no widely accepted criteria for functional LLIN survival is presently available but is in consideration.

3.3.5 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 products in the field. If mortality in the WHO cone bioassays falls below 80% and or knockdown falls below 95%, nets will be tested in a tunnel. If mortality in the tunnel test falls below 80% and blood feeding inhibition falls below 90%, the net will be considered to have failed to meet WHO criteria. If >20% of the nets sampled fail to meet the said criteria, the study will be stopped.

3.3.6 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, 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.

3.3.7 Data entry and analyses

Data entered into the computer will have only net IDs and not names of the net users such that they are not easily identifiable to the study participants. 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 shall be separately analyzed and reported.

Results of the insecticide susceptibility tests (tests using WHO tubes) will be analyzed for dose/response relationships (probit analysis) by the Maximum Likelihood method (Finney, 1971). The differences in mortalities will be compared between LNs 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 3.1.4.2.

3.3.8 Disease prevalence

Point prevalence of disease (malaria) in the two study arms, villages distributed with candidate LN and with positive control LN, should be assessed through blood surveys in each of the study arms. The sample size for blood survey should be estimated taking the immediate or average of the disease prevalence data available for the last three years in the study area. Minimum two blood surveys will be conducted, the first one, a month prior to LLIN distribution (base-line) and the second one at the end of the study. 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. Blood

A candidate LN is considered to meet the criteria for efficacy for testing in phase III evaluation, if after 3 years, at least 80% of the sampled nets are effective in WHO cone tests with ≥ 95% knockdown or ≥ 80% mortality. In the event of failure of nets as per knockdown and mortality criterion, tunnel tests are conducted and the criterion will be ≥ 80% mortality or ≥ 90% blood feeding inhibition. The net will be considered to have failed to meet WHO criteria if >20% of the nets sampled fail to meet the criteria, the study will be stopped. The candidate LN is considered to have met the WHO efficacy criteria based only on entomological parameters. The data on malaria prevalence and incidence can be additional parameters not amounting to the failure of the net.

sample from finger prick of the individuals in the selected households will be tested using RDK or microscopic examination. For collection of data, tabulation and analysis please refer to section 2.3.7.1.5.

Blood smears will be collected from RDT (bivalent)-negative patients and screened microscopically for malaria infection other than *P. falciparum* and *P. vivax*. For collection of data, tabulation and analysis please refer to section 2.3.7.1.5.

3.3.9 Disease incidence

Fever surveillance should be carried out in the villages with candidate LN and positive control LN during visits 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 (bivalent)-negative patients and screened microscopically for malaria infection other than *Plasmodium falciparum* and *P. vivax*. For collection of data, tabulation and analysis please see section 2.3.7.1.6. Data on malaria incidence from the national programme will also be included for the study.

4

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) so that the flying mosquitoes of the target species will get contacted with the droplets and killed (WHO, 2009) instantly, but space spraying lacks any residual effect. By killing adult mosquitoes, not only bites are prevented, but breeding is also contained, resulting in net reduction in the mosquito population. There are two types of space spraying,1) thermal spraying (volume mean diameter VMD or median mass diameter MMD, $Dv_{0.5} < 50$ μm) and 2). ULV cold spraying ($Dv_{0.5} < 25$ μm. $Dv_{0.5}$ (VMD or MMD) is the droplet size at which half (0.5) of the spray cloud volume is contained in droplets that are smaller (and by inference, half the volume in droplets are larger). 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, used in public health pest control programmes against nuisance mosquitoes and flies and during epidemics/outbreaks for control of vector-borne diseases in public health.

For new insecticide molecules Phase I evaluation will be carried out in the laboratory. WHO-Pre-qualified products that have, the products undergone Phase I evaluation by WHO recognized/ GLP certified labs or WHO Collaborating Centres following WHO guidelines are exempted from Phase I evaluation.

This 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 disease vectors.

The NCVBDC recommends thermal fogging during disease outbreaks/epidemics, both indoors and outdoors to knock down/kill the infected vector population and 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 ($Dv_{0.5}$)] and flight activity of the targeted vector.

4.1 Phase I: Laboratory evaluation

Duration: 6 months

Objectives

- To determine intrinsic activity of the insecticide
- To establish discriminating concentrations

- To assess cross resistance to other insecticides
- To determine insecticidal activity of technical grade insecticide/formulation
- To determine intrinsic activity of the insecticide and its discriminatory concentration for monitoring resistance and cross resistance to other insecticides will be carried out as described under sections 2.1.1.; 2.1.2 and 2.1.6.

4.1.1 Determination of insecticidal activity of the technical grade insecticide/formulation

The objective is to (i) determine LC_{50} and LC_{99} of the technical grade insecticide or its formulation against the target vector and (ii) the effective dosages of application in field.

In this test, the target species susceptible to the candidate insecticide (technical grade insecticide/formulation) is exposed to test concentrations of the atomized insecticide in a 'Wind tunnel' (Figure 4.1). Generally, six concentrations in μg/ml yielding mortality range between 5% and 100% should be tested, which comprise one concentration gives 100% mortality, at least two gives between 50% and 99% mortality, one gives around 50% mortality and at least two concentrations give between 5% and 50% mortality. For each test concentration, cages of mosquitoes, each containing 25 non-blood-fed, 2–5-day-old susceptible female mosquitoes are used. A total of 100 mosquitoes (25 mosquitoes × 4 replicates) are required for each test concentration and 50 mosquitoes (25 mosquitoes × 2 replicates) for the control. The mosquitoes are exposed to one of the test concentrations of the atomized insecticide in a wind tunnel.

Table 4.1. Determination of susceptibility to the technical grade insecticide/ formulation (wind tunnel test)

Date of Test: .....Temp: Min ..... Max: .....Humidity: Min .....Max .....

Test species: ..... Lab/F1/Field collected: .....Insecticide concentration: .....

Exposure: Start time: ..... End time: .....Holding: Start time.....End time: .....

Test done by: ..... Supervised by: .....

Replicate	No. exposed*	No. knocked down in 1 h	% knockdown	No. dead after 24 h	% Mortality	% Corrected mortality#
Treated 1						
Treated 2						
Treated 3						
Treated 4						
Treated total						
Control 1						
Control 2						
Control total						

*25 mosquitoes per 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).

$$\% \text{ Corrected mortality} = \frac{(\% \text{ Treated mortality} - \% \text{ Control mortality})}{(100 - \% \text{ Control mortality})} \times 100$$

Description of 'Wind tunnel'

The apparatus consists of a cylindrical tube (15.2 cm 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 (15.2 cm internal diameter and 2.5 cm depth). 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 (Figure 4.1 & 4.2). For details of equipment specifications, maintenance and procedure, please see Annexure 3 in WHO (2009).

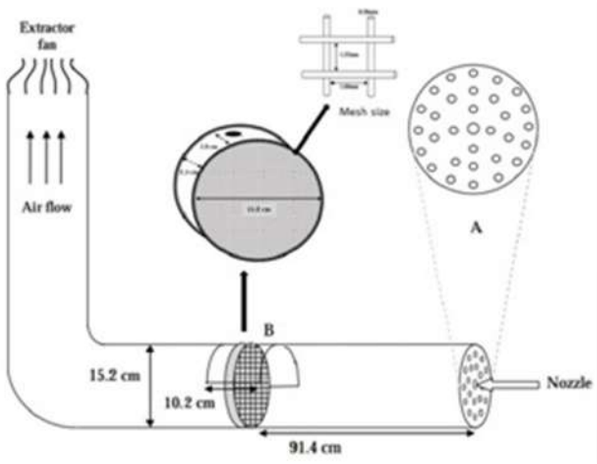

Figure 4.1: Schematic diagram of wind tunnel (Source: WHO, 2009)

Figure 4.2: Sealed cage being positioned in wind tunnel (Source: WHO, 2009)

Procedure

The insecticide (technical grade) dissolved in 0.5 ml acetone solution is atomized through a nozzle (that takes approximately 3 seconds) to produce droplets with a $Dv_{0.5}$ of 15 ± 2 μm at the position of the cage containing test mosquitoes. Mosquitoes are held in the wind tunnel for a further 5 seconds. After the test, the mosquitoes are transferred to pre-labelled clean holding cups covered with nylon net on the rim fastened with rubber band. Cotton swabs with 10% glucose solution are placed on the nylon net. The cups will be held in a dark place for 24 h at 27 ± 2°C temperature and 80% ± 10% RH. The mortality is recorded after 24 h. Prior to testing of insecticide, control tests should be done using acetone followed by lowest to higher concentrations of the insecticides. Between the tests with each concentration, the wind tunnel is cleaned with a spray of 0.5 ml acetone.

The test should be repeated three times using three separate batches of mosquitoes and the results are combined for log-dose probit regression analysis of concentration and mortality. The LC_{50} and LC_{99} values 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 (Table 4.1).

Wind tunnel procedure can also be used to test efficacy of the space spray formulations. But appropriate droplet size should be ensured by using appropriate nozzle. Wind tunnel testing is not suitable for most high-volume thermal fog formulations (WHO, 2009).

4.2 Phase II: Small-scale field evaluation- Indoor (human dwellings) and outdoor

Duration: 6 months

Objectives

- To determine the efficacy of the given insecticide formulation for space spraying in indoors and outdoors
- To determine the optimum field application dosage of the insecticide formulation for space spraying in indoors and outdoors

Small-scale evaluation in field is conducted to assess the efficacy of the space spray formulation on laboratory-reared mosquito species. 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, moving speed of spray-man, nozzle angle and pressure need to be standardized. Model calculation for application of space spray based on the flow rate of the sprayer and speed of application of applicator or vehicle is given in Appendix 2. Optimal conditions for outdoor spraying prevail when there is an increase in temperature with height (inversion, with stable conditions that help to keep the small droplets from rising above the target zone, zone of mosquito flying activity) and when the temperature is compatible with the diel flight activity of the target species. Favorable meteorological conditions usually occur at or near sunset (dusk hours), throughout the night, and up to an hour after sunrise (dawn) (WHO, 2009). 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 $Dv_{0.5}$ of desirable size, usually 15 $\pm$ 2 μm. In case of rapid volatility or other physical characteristic of the test material, the droplet size with $Dv_{0.5}$ of 10–40 μm may be used.

4.2.1 Outdoor applications

In the small-scale outdoor trial, 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 (Figure 4.3). The cages are placed at 25, 50, 75 and 100 m downwind of the spray perpendicular to line of application of insecticide. The dosage of active ingredient per hectare that causes at least 90% mortality is determined.

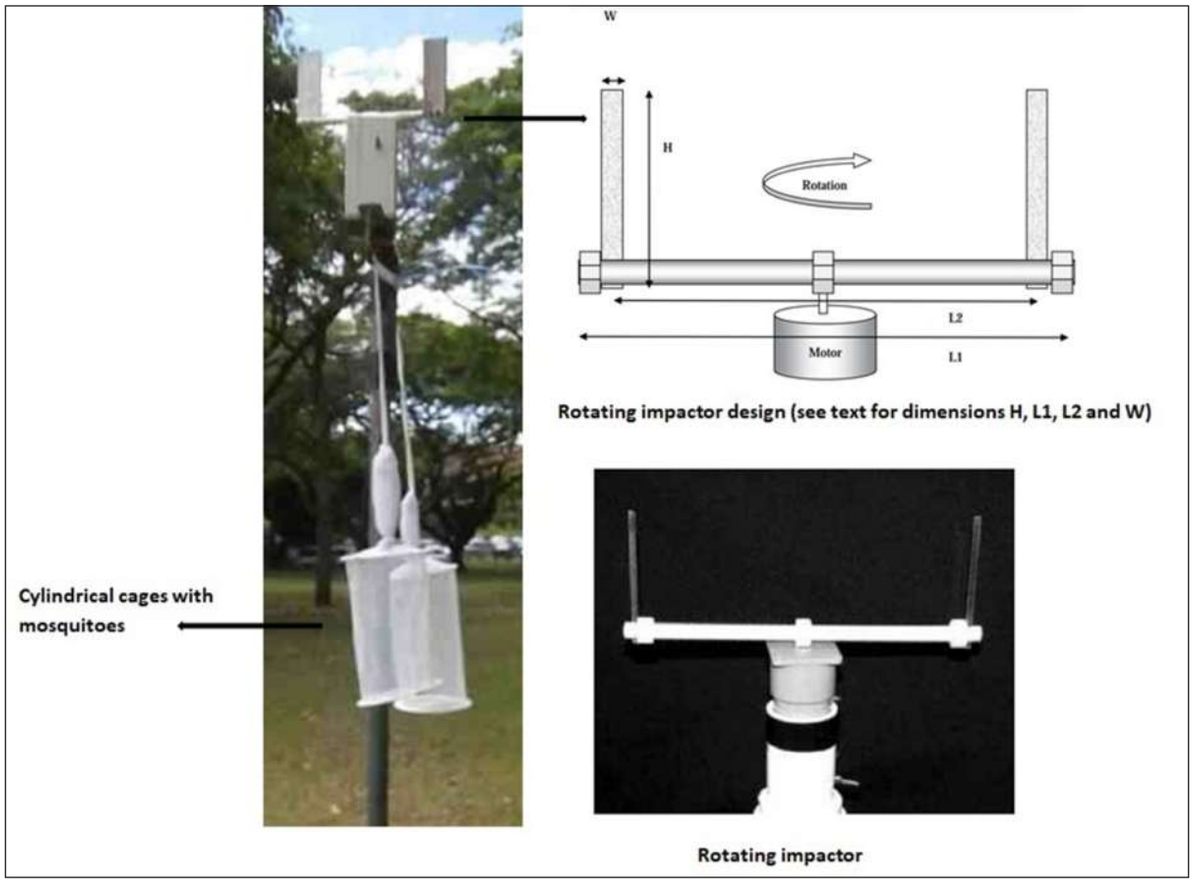

Figure 4.3: Cylindrical cages with rotating impactor, (Source: VCRU, USM, Malaysia) design and photograph of rotating impactor (Source: Annexure 8 in WHO, 2009)

4.2.2 Physical characterization of spraying

To physically characterize the spraying, the droplet size is assessed. Determination of droplet size and density at the selected sampling sites is an important parameter on which the effectiveness

of the insecticide space spray depends. Different techniques are available such as laser-based techniques, hot-wire anemometry and slide-wave and rotating collector impacting technique. Former two techniques are not appropriate for field use as laser-based technique is laboratory-based method while the latter is not suitable for thermal fogging studies. The slide-wave and rotator technique is found suitable for field and is field-friendly.

Silicone/Teflon/magnesium oxide coated slides are placed on rotators (Figure 4.3) kept adjacent to selected cages to measure the droplet density and droplet size of the spraying (see Annexes 7 and 8 in WHO, 2009). Prior to spraying, the rotator is switched on. Fifteen minutes post -spraying, the rotator should be switched off and the slide collectors removed immediately andat the same time the bioassay cages are also removed. The slide collectors are then placed in a protective holder and transferred to the laboratory for droplet size assessment as soon as possible.Simultaneously, slide collectors should also be placed with the control cages in an unsprayed control are kept in parallel at least 50 m upwind 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, slide 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 $< 10\ \mu$m. The craters in magnesium oxide/silicone- or Teflon-coated slides are examined under a microscope and the droplet size is measured. The $Dv_{0.5}$ and $Dv_{0.9}$ are calculated (see Annexure 8 in the WHO, 2009). For each collector, a minimum of 200 droplets should be measured reading across the width of the collector as many times as required.

After collection of appropriate number of droplets on the size and measurement the droplet density may be determined as described in Annexure 9 in WHO (2009).

4.2.3 Dosage determination

In small-scale field trials, a range of dosages of the given insecticide are tested based on 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 dosage between 80% and 95%. It would be appropriate if the sequence of dosages is randomized. The spray equipment is cleaned with acetone between every application. For each dosage, 1-2 cages will be placed at four distances i.e., 25, 50, 75 and 100 m in a row (sample line) and a minimum of three such rows should be maintained at downwind (Figure 4.4). 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 comparator (insecticide in use in the national control programme) or a WHO PQT/VCP recommended insecticide for space spraying can be used as a positive control.

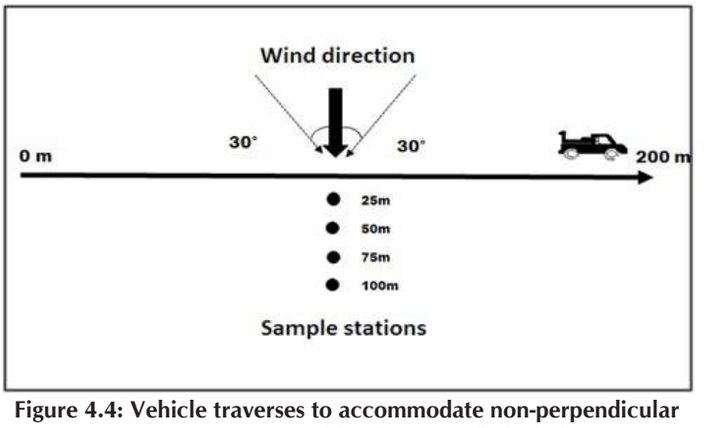

Figure 4.4: Vehicle traverses to accommodate non-perpendicular wind direction up to 30° (Source: WHO, 2009)

The trial should be conducted in open areas either during dusk hours or within an hour of sunrise (dawn) and sky should be clear and not rainy. The area of application should not have vegetation 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 increase 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 by15.4% 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 days-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 by distance, and provided with 10% glucose solution on a cotton wool. It should be ensured that from all the cages mosquitoes should be removed and transferred to the holding cups immediately 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 $\pm$2°C temperature and 80 $\pm$ 10% RH and the number dead at 24-hour post-spraying is recorded. If the tests are repeated on the same day, there should be a minimum of 30 minutes interval between each test, exposing mosquitoes obtained from different rearing batches. During each test, fresh dilutions of the formulation should be prepared and used. If the control mortality is between $\geq$5% and $\leq$20%, the test mortality should be corrected using Abbott's formula. For each dosage the average mortality of the replicates at each distance is estimated and recorded. 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 4.2.

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 phase III field evaluation. If the control mortality is >20%, the test should be discarded and repeated. If the control mortality is between $\geq$5% and $\leq$20%, the test mortality should be corrected using Abbott's formula.

Table 4.2: Knockdown and mortality of mosquitoes exposed to different concentrations of the insecticide formulation at different distances

Insecticide tested: .....Insecticide Dosages: ..... Distance of cage: .....Test date:

..... Temperature: ..... Min.....Max..... Relative humidity: Min: .....

Max.....Test species.....Lab/F1: ..... Test done by..... Supervised by.....

Replicates No.	No. of mosquitoes	No. knocked-down after 1 h	No. dead after 24 h	% knocked down after 1 h	% Mortality after 24 h	% Corrected Mortality
1						
2						
3						
Control 1						
Control 2						

$$\% \text{ Corrected mortality} = \frac{(\% \text{ Treated mortality} - \% \text{ Control mortality})}{(100 - \% \text{ Control mortality})} \times 100$$

4.2.4 Indoor applications

In the indoor application the dosage of active ingredient of the insecticide/formulation per cubic meter that causes at least 90% mortality is determined. Evaluation of indoor space spraying is donebased on mosquito mortality observed in the test cages in an empty room with a minimum volume of 30m^3. 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 $Dv_{0.5}$ of 15 ± 2 μm. At least three dosages of the insecticide should be tested. For each dosage, a minimum of three replicates (rooms) should be kept, using mosquitoes from different rearing batches. Fresh dilutions of the insecticide 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 cylindrical cage, the mosquitoes are quickly transferred to clean cups fastened with nylon nets that are labelled separately and provided with a 10% glucose solution on cotton wool on the nylon net for the mosquitoes to feed. While transferring the mosquitoes from the test cages to the holding cups, observation should be made on the number of mosquitoes knocked down in each cage. To record the mortality at 2 h post-spraying, the mosquitoes are maintained at 27 ± 2°C temperature and 80 ± 10% RH (Table 4.3). 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 exposures should be made parallel in separate rooms for each dosage of insecticide application. This is done prior to spraying by exposing mosquitoes in cages for 60 minutes at each of the designated 10 positions mentioned above. Before spraying, if the test mosquitoes held in a cage in the room (without ventilation) for one-hour show >10% knockdown or mortality, the rooms should be declared contaminated or unfit for testing.

Table 4.3. Observed knock down and mortality rates in exposed mosquitoes in cages indoors

Date of testing:..... Temperature: Min.....Max..... Relative humidity: Min .....

Max.....Test species: .....Lab/F1: .....Insecticide tested: .....Insecticide

concentration:.....

Cage No.	No. of mosquitoes exposed	No. knocked-down after 1 h	No. dead after 24 h	% knocked down after 1h	% Mortality after 24h	% Corrected Mortality
1. Treated 1						
2. Treated 2						
3. Treated 3						
4. Treated 4						
5. Control 1						
6. Control 2						

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 using Abbott's formula given below:

$$\% \text{ Corrected mortality} = \frac{(\% \text{ Treated mortality} - \% \text{ Control mortality})}{(100 - \% \text{ Control mortality})} \times 100$$

4.2.5 Human safety

Information on side effects/adverse events perceived by the insecticide handlers and spray-men will be recorded using a semi structured questionnaire (Annexure 12). The biochemical and the clinical parameters will be assessed pre- and post- spraying for the insecticide handlers and spray-men by a registered medical practitioner. Clearance from human ethics committeewill be obtained for the study (Annexure 1).

4.3 Phase III: Large-scale field evaluation

Duration: 12 months

Objectives

- To confirm the effectiveness of the application dosage (required to achieve a minimum of 90% control) under operational settings and
- To assess the impact of the space spraying on adult density of the target vector species.

Based on the results of small-scale field trials, the effectiveness of the space spray product/formulation should be assessed against field populations of the target vector species in operational settings. The trial should be a multi-centric study carried out at three eco-epidemiological settings. In each setting, the trial is repeated three times.

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 4.2) should be recorded and delivery characteristics of the insecticide should also be standardized (Section 4.2). In addition, topography of the study area, type 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 dosage(s) for conducting operational trials should be based on dosage(s) recommended on the manufacturer's label or the optimum dosage(s) that caused at least 90% mortality in small-scale trials. Spraying outdoors are based on dosage per hectare and indoor applications are based on dosage per cubic meter. 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, 2022). Spray equipment must be calibrated to deliver a spray droplet distribution with a $Dv_{0.5}$ of 15 $\pm$ 2 μm. In case of rapid volatility or other characteristics of the test material, larger or smaller droplets ranging from 10–40 μm ($Dv_{0.5}$) 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 favorable (Figure 4.5). Optimal conditions for outdoor spraying prevail when there is an increase in temperature with height (inversion, with stable conditions that help to keep the small droplets from rising above the target zone, zone of mosquito flying activity) and when the temperature is compatible with the diel flight activity of the target species. Favorable meteorological conditions usually occur at or near sunset (dusk hours), throughout the night, and within an hour of sunrise (dawn) (WHO, 2009). Preferred times of application are dusk. The space spray treatments should be dusk or dawn planned in such a way that it should coincide with the peak flight activity of the target species.

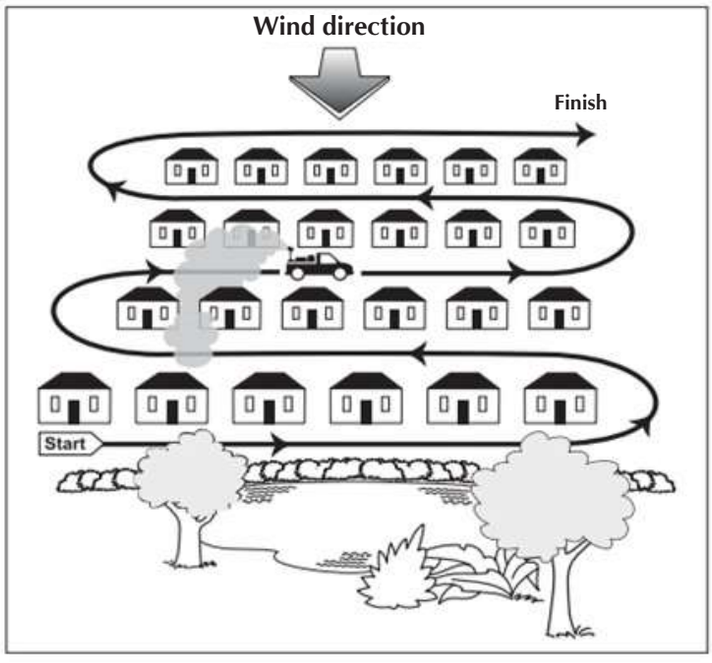

Figure 4.5: Spray application route relative to wind direction. Coverage is from downward to upwind. The first swath targets flying adults in the proximity to the breeding sites (Source: WHO, 2009)

4.3.1 Trial outdoors

4.3.1.1. Evaluation methods

The design of the trial should consider flight range and behavioral characteristics of the target species e.g., indoor resting (endophily) and/or outdoor resting (exophily), and the method of spraying. An adequately larger area (approx. one sq.km) 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 area. 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 order to monitor the relative changes in the density of the target vector species, appropriate adult sampling methods/devices should be used.

4.3.1.2 Evaluation using sentinel cages

The spatial diffusion of spray cloud in the test area is verified by observing mortality of mosquitoes confined in cages placed in sentinel sites selected in the sprayed areas (test area) up to the distance where the spray cloud is expected to reach. Batches of 25 non-blood-fed, laboratory reared, 2–5 days old insecticide-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.

One sampling site should be selected from an unsprayed area 50 m upwind direction of the treatment zone for control 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 $27 \pm 2°C$ and $80 \pm 10\%$ RH. Mosquitoes should be provided with 10% glucose solution-soaked cotton wool placed on the top of the cup. Percent knock down should be scored at 60 min after the exposure and %mortality after 24 h holding period. Along with the sentinel cages rotating slide collectors should be fixed to assess the droplet size and density.

4.3.1.3 Entomological evaluation

The entomological collections should be carried during pre-spray and post-spray periods to assess the impact of space spray on the wild populations of mosquitoes. Pre-spray collections should be made at least on three occasions leaving a gap of two days between successive collections and post-spray collections will be made on day 1, day 3 and day 7 post spraying or till the pre-spray densities are recorded to determine the period of loss of efficacy of the space spraying.

4.3.1.4 Mosquito collection indoors and outdoors

Resting mosquitoes in the house are collected using a mechanical/prokopack aspirator. Care should be taken to collect resting mosquitoes of all genera and species. Collections are made indoors in 3 fixed and 3 random houses and 3 cattle sheds (if available) in the locality by spending 10 min in each structure and simultaneously outdoor collections will be made from potential resting sites such as bushes, crevices, tree-holes/hollows, damaged walls, underneath the tunnels etc., for one hour in each area. Collections are also made from unsprayed areas for comparison. Mosquitoes are identified to species. The collections of mosquitoes are labelled according to the dosage/dosages and kept in 150 ml/ 300 ml cups (10 individuals per cup), with 10% sucrose solution and in a climatic chamber or in a room for 24 h maintained at 27°C ± 2°C and 80% ± 10% RH. The percent mortality after 24 h is recorded. Observation on number of dead mosquitoes after 24 h of holding provides data on mortality (Table 4.4). The mortality in treated and control areas is compared without adjusting as the impact of intervention in field is dependent on various factors (Table 4.4).

Table 4.4: Hand catch to assess the impact of space spraying on mosquitoes

Village/locality:..... District / city/ward: .....

State: .....Date of collection: ..... Insecticide & Dose:

.....Spray Round: .....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								
Dosage 4								
Control 1								
Control 2								

UF = Unfed; BF = Blood fed; SG = Semi-gravid; G = Gravid

*Unfed mosquitoes should be dissected for parity (Refer section 4.3.1.5).

Man Hour Density (MHD) – (No. of mosquitoes collected/No. of persons) × time (h).

4.3.1.5 Parous rates

The ovaries of all unfed mosquitoes collected by different methods should be dissected out and the tracheolar skeins observed to determine the parity (Table 4.5).

Table 4.5: Parous rates of the mosquitoes from hand catch collections

Village/locality: ..... Sub-centre: ..... PHC/CHC: .....

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) × 100

4.3.1.6 Light traps (LT)

The traps are set indoors at a height of about 2m from ground (in the selected human dwellings or cattle sheds) near the bait as well as outdoors during dusk hours at fortnightly intervals in both treated and untreated area. One LT each in two of the selected houses and cattle sheds (if cattle sheds are not available, another two houses will be selected for LT collection). The next morning, the trapped mosquitoes are collected, identified to species and recorded in the format given in Table 4.6. The density of species-wise mosquitoes is expressed as number/ trap-night.

Table 4.6: Light trap collections of mosquitoes outdoors

Village/locality:.....District/city/ward:.....State:.....

Insecticide/formulation: .....Dosage: ..... Date of collection: .....

Temperature: Min: .....Max..... RH: Min ..... Max .....

Trap No.	Collection site - No. & Type	Number collected		Gonotrophic condition			
		♂	♀	UF	FF	SG	G
01							
02							
03							

Note: Separate table to be prepared for each mosquito species
UF: Unfed; FF: Fully-fed; SG: Semi-gravid; G: Gravid

4.3.1.7 Data analysis

Data obtained from the sentinel cages and droplet collectors will be used for the assessment of dispersion/penetration of the insecticide. Average mortality of mosquitoes in the sentinel cages in a given area and the variation in the mortality between the cages should be estimated. To assess the efficacy of outdoor space spraying, the percentage reduction of wild caught mosquito populations between pre- and post-spraying and between sprayed and unsprayed areas will be estimated and compared using appropriate statistical method(s).

4.3.2 Space spraying indoors

Space spraying indoors will be carried out by selecting at least one village or one ward (in urban areas) with an approximate population of 3000. Spraying will be done covering all rooms in the houses. Efficacy of space spraying is assessed by selecting a minimum of 10 households selected across the sprayed area.

4.3.2.1 Evaluation using sentinel cages

In each household, at least three cages, one should be placed near the center of a room at 1.5 m height and the other two cages should be placed adjacent to preferential mosquito resting sites. Twenty-five (mixed age) wild caught female mosquitoes are released into each cage and exposed to insecticide application for 60 minutes in both experiment and control houses. 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. After the exposure of mosquitoes for 60 minutes, the cages are removed from the houses. The cages should be handled carefully wearing adequate protective clothing. The mosquitoes are quickly shifted from the cages to clean separately marked holding cups fastened with nylon net on the rim provided with a cotton wool soaked in 10% glucose 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 inhabitants into the house. The mosquitoes in the holding cups are maintained at $27 \pm 2°C$ and 80 $\pm$ 10% RH and mortality is recorded at 24-hour post-spraying.

Entomological assessment to compare pre- and post-spraying mosquito densities, parity and infection rates are to be carried out as described in section 4.3.1.3 (outdoor applications)

4.1.1.2. Data analysis

Average mortality (with standard deviation) is calculated for each dosage tested 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 $\geq 5\%$ and $\leq 20\%$, the test mortality should be corrected to the control mortality using Abbott's formula.

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 WHO Pre-qualified Vector Control Products and new insecticides. New larvicides are evaluated in three phases, Phase I, Phase II and Phase III.

5.1 Chemical larvicides

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

5.1.1 Phase I: Laboratory evaluation

The WHO-Pre-qualified products and the products undergone Phase I evaluation by WHO recognized/ GLP certified labs or WHO Collaborating Centres following WHO guidelines are exempted from Phase 1 evaluation.

Duration: 3 months.

Objectives

- to establish dose-response relationship of the larvicide against the target vector species,
- to determine LC_{50} and $LC_{99.9}$ values
- to establish a diagnostic concentration to discriminate between resistant and susceptible populations and dosages for application in Phase II trials
- to assess cross-resistance to the commonly used insecticides in vector control

5.1.1.1 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 in organic solvents (acetone or ethanol). 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 brown glass vial with Bakelite screw cap and covered with aluminum foil. Complete dissolution or dispersion of the material in the solvent should be ensured by vigorous shaking on a vortex mixture. Serial dilutions of the stock solutions (ten-fold) are made in ethanol or acetone (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 5.1). As given in the Table 5.1, 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 5.1. Aliquots of various strength solutions added to 100 ml water to yield final concentration

Initial solution		Aliquot (ml)*	Final concentration (PPM) in 100 ml
%	PPM		
1.0	10000.0	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
(Source: WHO/CDS/WHOPES/GCDPP/2005-13)

5.1.1.2 Laboratory bioassays

The LC_{50} and $LC_{99.9}$ values of the larvicide are determined from dose-response curves using laboratory colonized larvae of known age or F_1 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.

Figure 5.1: Laboratory evaluation of chemical larvicides

In laboratory bioassays, the activity range of the test material is determined first by exposing early IV instar larvae (25/replicate of 100 ml of different concentrations) 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 LC_{50} and $LC_{99.9}$ (Figure 5.1).

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 5.2) to 100 ml or 200 ml water in the cups. For each concentration, four replicates are set up with parallel controls (keeping equal number of replicates) in chlorine free tap water or distilled 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 h no larval food is required. However, food (finely ground dog biscuit and yeast powder 60:40 or fish food) may be provided if the exposure period is extended to 48 h 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 h, percentage mortality is calculated counting dead and moribund larvae together 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 5.2 (where, LC_{50} and $LC_{99.9}$ values and the outcome of slope and heterogeneity analysis are also recorded).

If more than 10% larvae pupate 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 as given below:

$$\% \text{ Corrected mortality} = \frac{(\% \text{ Treated mortality} - \% \text{ Control mortality})}{(100 - \% \text{ Control mortality})} \times 100$$

Table 5.2: Laboratory evaluation of the efficacy of larvicides against mosquito larvae

Experiment No: .....Treatment date: ..... Larvicide: .....

Test species: ..... Larval instar: ..... Temperature: .....

Photo period: ..... Test done by..... Supervised by.....

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

Conc. (mg/L)	Replicates	Number exposed	Dead/Moribund		% Mortality		% Corrected Mortality	
			24 h	48 h	24 h	48 h	24 h	48 h
	3							
	4							
T4	1							
	2							
	3							
	4							
T5	1	`						
	2							
	3							
	4							

UCL: Upper confidence limit

LC_{50} (UCL 95%) : _____

LC_{90} (UCL 95%) : _____

$LC_{99.9}$ UCL 95%) : _____

Slope: _____ Heterogeneity: _____

5.1.1.3 Data analysis

For determination of LC_{50} and $LC_{99.9}$ data from all replicates should be pooled. The LC_{50} and $LC_{99.9}$ are calculated from a log dosage-probit mortality regression line using a computer software programme or using a log-probit paper.

5.1.1.4 Determination of diagnostic concentration

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

5.1.1.5 Cross-resistance assessment

1. To assess cross-resistance to other insecticides that are currently in use in the programme, bioassays should be done using the diagnostic concentration of the test larvicide and of other larvicides in use for larval control.
2. The new candidate larvicide should be tested simultaneously against a small number of distinct, multi-resistant mosquito strains and a susceptible strain, according to the procedures outlined in Section 5.1. (For further details refer to WHO/CDS/WHOPES/ GCDPP/ 2005.13)
3. If cross-resistance is detected, its exact nature will be determined by testing the larvicide against strains that each possess a single resistance mechanism. The mechanism of resistance may be assessed following the procedures outlined in the WHO document, *Techniques to detect insecticide resistance mechanisms* (WHO 1998a).

5.1.2 Phase II: Small-scale field / Simulated field evaluation

Phase II evaluation of the larvicide is exempted if it is already evaluated in India for WHO Pre-Qualification.

The larvicides that show promising activity (preferably, $LC_{50} < 1$ppm) in laboratory evaluation (Phase I) are considered for small-scale field evaluation (Phase II). The dosages for Phase II trials

are calculated based on $LC_{99.9}$ values determined in Phase I trial. The application dosages will be determined by multiplying the $LC_{99.9}$ value with a factor of 2 and above so as to obtain at least a range of graded 3–5 dosages for small scale evaluation.

Duration: 6 months

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(s) for Phase III trial, and
- To record qualitative observations on presence/absence of the non-target organisms, especially predators, cohabitating the mosquito larvae

5.1.2.1 Evaluation 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, plastic barrels, garden pits, pools, rice plots, river/ stream bed pools, and wells; for *Culex* species, stagnant drains, cesspits, cesspools and disused wells and for *Aedes* spp., cement tanks, drums, peri-domestic water storage containers such as plastic barrels are best suited. A minimum of five 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 (Appendix 3). As far as possible, the habitats selected should be similar and comparable in terms of vector density. Each of the confined breeding habitats or containers can be considered as a discrete habitat or replicate. Habitats such as drains, irrigation canals, rice fields, ponds may be divided into sectors of 10 m length and replicated for treatment and control. Stagnant drains/ canals (cement lined 'U' shaped) choked with silt and debris and had no connection with other drains/canals should be selected. Every segment of 10 m length of the drain/canal is considered as a replicate. However, while applying the formulations, the entire length of the drain should be treated. Separate drains are selected for each dosage/formulation and for 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.

Figure 5.2: Monitoring of larval and pupal densities in stagnant drain

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 (Figure 5.2). 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.

Three to five 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. Hand held compression (1-liter capacity) sprayers may be used for application of larvicide formulations in container habitats such as barrels, cement tubs, etc (Figure 5.3).

Figure 5.3: Application of chemical larvicide using hand compression sprayer in drains

In container habitats with *An. stephensi* and *Ae. aegypti* breeding, the dosages should be applied considering the volume of water in the habitats. In larger water habitats such as street drains, abandoned wells, cesspools, the dosages should be applied based on the surface area of the habitats.

1. The following formula is used to determine the application rate:
2. Rate of application (ml/m) = Flow rate (ml/min)/ Width of swath (m) × Walking speed (m/min)

3. The required concentration of larvicide suspension is calculated as follows:

4. Concentration of larvicide (emulsion/suspension) = [Dosage to be applied (g/m^2) / Application rate (ml/m^2)] × 100

5. In the third step, preparation of working solution was done using the following formula:

   X = (A/B)-1

   X = parts of water to be added to 1 part of larvicide

   A = concentration of EC formulation

   B = concentration of larvicide suspension (working solution)

Other formulations such as pellets, tablets and briquettes can be manually broadcast or thrown in the water. The granular formulations can be evenly applied over each habitat manually. In larger habitats small hand dispenser (1.5–2.0 liter capacity) can be used (the dispensers should be calibrated prior to application for uniform and accurate dispensation of the granular formulation).

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 untreated control habitats. 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 prior to 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 5.3.

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 control habitats using Mulla's formula:

$$\% \text{ Reduction} = 100 - \frac{C1x\ T2}{T1x\ C2} \times 100$$

Where,

C_1 = Pre-treatment larval/pupal density in control habitats

C_2 = Post-treatment larval/pupal density in control habitats

T_1 = Pre-treatment larval/pupal density in treated habitats

T_2 = Post-treatment larval/pupal 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. Pairwise comparison of dosages is done using the post-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.

5.1.2.2 Trial in simulated field condition

In situations, where adequate number of larval habitats with comparable densities are not available for treatment and control, trials may be conducted in stimulated field conditions. Testing is done in

Table 5.3: Field evaluation of larvicides against mosquito larvae

Experiment No: ..... Locality: ..... Street: ..... Habitat type : .....

Larvicide formulation : ..... Species : ..... Treatment date: .....

Investigators: .....

Date	Days Pre/ post-treatment	Test Dosage/ control	Replicate No.	Sample No.	Larval instars & pupae (P)							No of NTOs	Water Temp	pH	Surface area	Remarks
					I	II	I + II	III	IV	III + IV	P					

NTO: Non-target organism

simulated conditions against the mosquito species that breed in domestic and peri-domestic clean water habitats. Trials can be carried out in containers (cement tubs/tanks, plastic barrels, drums, jars, buckets, tubs, etc.) *Cx. quinquefasciatus*, testing is not done in simulated field condition, as this species is known to breed in polluted water habitats.

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 litres or 200 litres 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 litres or 200 litres). The tubs are screened with nylon mesh to prevent egg laying by other mosquitoes or insects and to protect the water from falling debris (Figure 5.4)

Figure 5.4: Evaluation of chemical larvicides in plastic barrels under simulated field conditions

Two regimens of water can be used: In the first regimen, tubs are kept full for the entire 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 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 a range of graded 4–5 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 fourth 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 format as shown in Table 5.3.

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.

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 post-treatment counts (% reduction is calculated using Mulla's formula). For analysis of data, the method described under section 5.1.2.2 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 at 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 Phase III trial.

5.1.3 Phase III: Large-scale (village scale) field evaluation

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, covering all seasons.

Duration: 18 months

Objectives

- To confirm the efficacy of the larvicidal formulation applied at the selected optimum field application dosage(s) against the target vector species in natural breeding habitats
- To confirm the residual activity and application intervals (frequency) in clean and polluted breeding habitats
- To record observations on the ease of application and dispersal of the larvicidal formulation
- To record residents'/community acceptance
- To document any perceived side-effects on operators and
- To observe the effect of the treatment on co-habiting non-target organisms

5.1.3.1 Selection of study area

Two comparable urban or rural areas are selected for the trial, one for treatment and the other for control. All types of breeding habitats in the selected areas are surveyed to ascertain the breeding of the target vector species in order to decide the suitability of the localities for the evaluation. For this purpose, larval/pupal samples will be collected using an enamel dipper (300 ml capacity) from different types of habitats and allowed to emerge in the laboratory. The emerged adults will be identified to species using morphological identification keys. It is to be ensured that the areas selected for treatment and control should have adequate number of larval habitats of the target vector (*Aedes*/*Anopheles*/*Culex*) species with comparable densities. Species like *Culex quinquefasciatus* breeds in polluted water with rich organic content, whereas *Anopheles* and *Aedes* species breed in relatively fresh water habitats. Separate trials should be undertaken

for the species breeding in clean or polluted water habitats using the respective optimum field application dosage (s) determined in phase II evaluation.

Selection of larval habitats should be done carefully considering the residual activity of the formulations (as determined in phase II evaluation). Accordingly, the habitats which are likely to retain water for longer duration are selected. In other habitats with small volume of water, source reduction activities should be done. During the evaluation period, many domestic /peri-domestic habitats are likely to get disturbed by frequent washing and other related activities by the residents. Considering all practical constraints, a minimum of 30 replicates of each type of major habitats (available in adequate numbers) should be selected for treatment and equal number of replicates should be kept untreated for control. All the other different types of larval habitats of the target vector species available in the trial area should be included for treatment and control. As in the case of small-scale field trials, each of the confined breeding habitats such as cesspits, borrow pits, cement tanks, wells can be considered as a discrete habitat or replicate. Habitats such as drains, irrigation canals, rice fields, ponds may be divided into sectors of 10 m length and replicated for treatment and control.

5.1.3.2 Assessment of density prior to treatment

Prior to treatment, density of larvae and pupae should be assessed in treatment and control habitats on at least two occasions during a week. The immature density should be measured in different types of habitats using appropriate sampling devices (as given in section 5.1.2.1).

5.1.3.3 Application of larvicidal formulation

All larval habitats in the treatment area should be treated at the optimum field application dosage determined in Phase II trial, using the equipment appropriate to the formulation The optimum dosage for the major larval habitat of the target species in the area can be used for all the habitats. In small-scale trial, if there is a wide variation between the dosages for each type of habitat, the specific dosage should be applied to each type of habitat. It is to be ensured that all field workers and supervisors are to be blinded as to the allocation of treatments to avoid bias during the trial.

The vector species (*Cx. quinquefasciatus*) that breeds in perennial habitats, the entire trial will be repeated/replicated three times covering different seasons, such as summer, rainy and winter. In most of the epidemiological settings in India, profuse breeding of *Ae. aegypti* is observed during rainy season (June to September) the phase III trials against such species is mainly done during the rainy season. In other seasons, adequate number of habitats may not be available for the trial. Hence, number of re-treatments may be restricted depending upon the residual activity of the formulations and availability of larval habitats. The habitats will be retreated at the frequency of applications based on the residual activity determined in phase II trial during the given season.

In container habitats with *An. stephensi* and *Ae. aegypti* breeding, the dosages should be applied considering the volume of water in the habitats. In larger water habitats such as street drains, abandoned wells, cesspools, the dosages should be applied based on the surface area of the habitats.

5.1.3.4 Assessment of density after treatment

The post-treatment monitoring of the density of larvae and pupae in all the treated and control habitats should be carried out by taking fixed number of samples 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 format (Table 5.3).

5.1.3.5 Effect on non-target organisms

For assessing the impact of the larvicidal application on non-target organisms that co-habitat with mosquito immatures, 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-habit mosquito larvae.

5.1.3.6 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. In the treatment area, baseline data should be collected from a random sample of households prior to larvicide treatment. Acceptability of larvicide treatments should be assessed on week 1 and 2 post-treatment. A qualified social scientist should be engaged to develop a culturally sensitive questionnaire, that should be pre-tested before use. For conducting interviews, clearance should be obtained from the human ethics committees of the respective institutions/ authorities. The informed consent form and the information sheet containing the study details to be provided to the residents should be approved by the ethics committee.

5.1.3.7 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 5.1.2.2. will be followed to confirm the residual efficacy and frequency of application.

5.2 Bacterial Larvicides

Bacillus thuringiensis subsp. *israelensis (Bti)* 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 contains the mosquito larvicidal toxin called delta-endotoxin which is thermostable up to 47°C. This bacterium is lethal to mosquitoes as well as black flies. It has been found to have high toxicity against *Culex, Anopheles* and *Aedes* larvae in different habitats. The endotoxin of this bacterium is found to be safe to humans and to domestic and other aquatic organisms. Different formulations of *Bti* are available (Figure 5.5). Any new formulation of this bacterium as well as new bacterial agents that are found to be safe will be evaluated as described below.

5.2.1 Phase I: Laboratory evaluation

Duration: 3 months

Objectives

- to establish dose-response relationships of the given bacterial larvicide(s) against the target vector species,
- To determine LC_{50} and $LC_{99.9}$ values and field application dosage(s) for Phase II trial

5.2.1.1 Preparation of stock solution and test concentrations

For conducting the 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 brown glass vials and frozen for future bioassays. The frozen stock solution should be homogenized completely before serial dilutions are made.

The stock solution is 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 5.2).

Figure 5.5: *Bacillus thuringiensis* subsp. *israelensis (Bti)* formulations: (a) Water dispersible powder, (b) Briquette and (c) Granular (d) Liquid formulation

5.2.1.2 Laboratory bioassays

The laboratory bioassay procedures for bacterial larvicides are the same as those for chemical larvicides (Please refer to section 5.1.1.2). Mortality is scored at 24 h post-treatment for the bio-larvicide or upto 72 h post-treatment by counting the live larvae remaining in the test cups. For exposure period of 24 h, addition of larval food to the test cups is not required and for extended periods, food should be added. The results are entered in the given format (Table 5.2).

5.2.1.3 Data analysis

LC_{50} and $LC_{99.9}$ values of the candidate biolarvicide will be calculated from the dose-mortality regression lines by probit analysis. The dosages for Phase II trials are calculated based on $LC_{99.9}$ values determined in Phase I trial.

5.2.2. Phase II: Small-scale field evaluation

Bacterial larvicides that show promising (preferably $LC_{50} < 1$ppm) activity in the laboratory studies (Phase I) are subjected to small-scale field evaluation (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 graded 3–5 dosages.

Duration: 6 months

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.

5.2.2.1 Trial in natural breeding habitats

The small-scale dose determination trial procedures for bacterial larvicides are the same as described for chemical larvicides (Section 5.1.2.1)

5.2.2.2 Data analysis

Refer to section 5.1.2.2

5.2.2.3 Trial in simulated field condition

The trial procedures in simulated field conditions for bacterial larvicides are the same as described for chemical larvicides (Section 5.1.2.3)

5.2.2.4 Data analysis

Refer to section 5.1.2.4

5.2.3 Phase III: Large-scale field evaluation (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, covering all seasons.

Duration: 18 months

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
- To observe the effect of the treatment on co-habiting non-target organisms

5.2.3.1 Evaluation

Selection of study sites, assessment of density prior to treatment, application of bacterial larvicide, assessment of post-treatment density, effect on non-target organisms, operator's and resident's acceptability and data analysis for phase III evaluation of bacterial larvicides are the same as those for chemical larvicides. Refer to sections 5.1.3.1 to 5.1.3.7.

5.3 Insect Growth Regulators (IGRs)

IGR compounds are of two types: (i) Chitin synthesis inhibitors (e.g., diflubenzuron, novaluron) affect the synthesis of chitin during molting 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 and (ii) Juvenile hormone mimics (e.g., pyriproxifen and methoprene) delay the molting process of larvae and pupae thereby increasing the inter-molting 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 percentage inhibition emergence (IE%).

5.3.1 Phase I: Laboratory evaluation

Duration: 3 months

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 99.9% inhibition of adult emergence (IE_{50} and $IE_{99.9}$)

5.3.1.1 Determination of IE_{50} and $IE_{99.9}$

Determination of IE_{50} and $IE_{99.9}$ of insect growth regulators is done on laboratory reared larvae of known age or F_1 larvae of the field collected adult mosquitoes. Bioassays are carried out following the WHO procedure (WHO/CDS/WHOPES/GCDPP/2005-13).

5.3.1.2 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 5.1.1.1 and 5.1.1.2).

5.3.1.3 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 escape of emerged adults. Mortality is scored every alternate day until complete emergence of adults. The treated and control replicates are maintained at 25–29°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 format (Table 5.4). Morphological deformities/ abnormalities, if any, of the molting larvae and pupae or the emerging adults should be recorded.

5.3.1.4 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 instar 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/ bio-larvicides, 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 IE_{50} and $IE_{99.9}$ values are estimated from a log dosage-probit mortality

Table 5.4: Laboratory evaluation of the efficacy of insect growth regulators (IGRs) against mosquito larvae

Experiment No: ..... Treatment Date: ..... IGR: .....

Test species: .....Larval instar: .....Temperature: .....

Lighting: ..... Test done by..... Supervised by.....

Conc. (mg/L)	Replicates	Number exposed	24 h*			48 h*			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...															
LC_{50} (UCL 95%):										LC_{90} (UCL 95%):						
$LC_{99.9}$ (UCL 95%):																
Slope:										Heterogeneity:						

L: Larvae; P: Pupae; A: adults; UCL: Upper confidence limit;

*Observations will be made beyond 48 h till complete mortality or adult emergence and additional columns to be added for recording the data

If more than 10% larvae pupate 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 as given below:

$$\% \text{ Corrected mortality} = \frac{(\% \text{ Treated mortality} - \% \text{ Control mortality})}{(100 - \% \text{ Control mortality})} \times 100$$

regression analysis using computer software programs or estimated from log-probit paper. The procedure for data analysis given in section 5.1.1.3 should be followed. The dosages for the Phase II trial are determined by multiplying the observed $IE_{99.9}$ value with a factor of 2 and above so as to obtain at least a range of graded 4–5 dosages for small scale testing.

5.3.2 Phase II: Small-scale field evaluation

Duration: 6 months

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 percentage 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 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 clean water container habitats (Table 5.5). 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:

$$\% \text{ Reduction} = [100 - (C1 \times T2)/(T1 \times C2)] \times 100$$

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.

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

Where,

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

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 5.1.2.2 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.

5.3.2.2 Evaluation 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 5.1.2.3) 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

Table 5.5: Field evaluation of insect growth regulators against mosquito larvae

Experiment No: ..... Locality: ..... Street: ..... Habitat type: .....

IGR formulation: ..... Species: ..... Treatment date: ..... Investigators: .....

Date	Days Pre/ post- treatment	Dosage/ control	Rep. No.	Sample No.	Larval instars & pupae (P)							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						

NTO: Non-target organism; Rep: Replicate

the residual activity, the treatments are challenged with new cohorts of larvae (early 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 format as given in the Table 5.5.

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 5.3.2.1);

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

Where,

C = percentage emerging or living in control habitats and

T = percentage emerging or living in treated habitats.

The mean number of larvae and pupae 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 5.1.2.2 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 5.1.2.4.

The effective duration (the post-treatment day up to which the lower limit of the 95% CI for the mean percentage reduction of density will be ≥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.

5.3.3 Phase III: Large-scale field evaluation (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 covering all seasons.

Duration: 18 months

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 (frequency) 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

5.3.3.1 Evaluation

Selection of study sites, assessment of density prior to treatment, application of IGR, assessment of post-treatment density, effects on non-target organisms, operational and residents' acceptability and data analysis for phase III evaluation of IGR are the same as those for chemical larvicides. Refer to sections 5.1.3.1 to 5.1.3.7. 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 5.3.2.1).

5.3.3.2 Effect on non-target organisms

The IGRs affect molting process of insects either by inhibiting chitin synthesis or prolonging the inter-molting 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 non-target insects, if present.

6

Monomolecular Films

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

6.1 Phase I: Laboratory evaluation

Duration: 3 months

Objective

- To determine the effective dosage for application of MMF and its efficacy against mosquito larvae and pupae

6.1.1 Determination of the effective dosage for application of MMF and its efficacy against mosquito larvae and pupae

In laboratory trials monomolecular film should be tested against the four larval instars and pupae. Different doses (0.1 to 1.0 ml/m^2) should be applied and tested starting from the lowest dosage. 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 at 6 different dosages in separate trays. The effective dosage (ml/m^2) is the lowest dosage 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 effective dosage, I/II, III/IV instar larvae and pupae will be tested separately keeping 3 replicates for each. In each replicate (tray), 100 laboratory colonized I/II or III/IV instar larvae or pupae should be released. For each group, two control replicates (100 larvae/pupae per replicate without MMF) should be run in parallel. Two hours prior to the introduction of larvae, liquid larval food should be added to the trays and stirred. The effective dosage of MMF will be applied to the test trays after the introduction of larvae/pupae. Observation on larval/pupal mortality in test and control replicates will be recorded at 24, 48 and 72 h and more, if required. The mortality data should be recorded in the format given in Table 6.1. The data will be subjected to log-probit regression analysis to calculate the LT_{50} and $LT_{99.9}$ in hours. In general, control of 1st to 4th instar mosquito larvae is relatively slower than control of pupae, and control of younger instars (I & II instar) is slower than older (III & IV instar). The test cups are maintained at a temperature range of 25–29°C and at a photoperiod of 12-hour light and 12-hour dark period (12L:12D).

Table 6.1. Observations on larval/pupal mortality in trays treated with the effective dosage of MMF

Experiment No: ..... Treatment date: .....MMF: .....

Test species: .....Larval instar/Pupae:.....

Temperature: ..... Photo period: .....Test done by: .....

Supervised by:.....

Immature stage	Number introduced	Mortality			Adult emergence	Remarks
		24h	48h	72h*		
I/II Instar						
III/IV Instar						
Pupae						

* if the observation is made beyond 72 hour additional columns may be added

The efficacy of MMF at its effective dosage will be decided based on the time point (in hours) at which 90% mortality of larvae and pupae is obtained in the trays.

6.2 Phase II: Small-scale field evaluation

Duration: 6 months

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 dosage(s) for Phase III trial.

6.2.1 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, borrow pits, pools, water fountains and disused wells; for *Cx. quinquefasciatus* stagnant drains, cesspits, cesspools and disused wells and for *Aedes* spp. tanks, drums, cement tanks, peri-domestic water storage containers and coolers are best suited. A minimum of 4 replicates should be randomly selected for each type of habitat and dosage. Equal number of controls 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 5.3.2.1). 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 habitat, while for stagnant drain dips at a distance of 3–5 m are recommended. The larval instars and pupae collected from each dipper/bucket sample are counted stage wise and returned back to the habitats.

To evaluate the effective dosage of the MMF at small scale in the field, larval/pupal mortality will be recorded at $LT_{99.9}$ as determined in Phase I laboratory studies, and at two, four or six times higher the $LT_{99.9}$. Thus, about three to five dosages (based on observation time) of MMF formulation, obtained from Phase I or manufacturers label instructions, are tested in small-scale field trials. 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 hours and later at weekly intervals until the density of IV instar larvae and pupae reaches a level comparable to that in untreated control habitats. Data are recorded in the format given in a Table 6.2.

Table 6.2. Density (mean no. of 5 dips) of *Anopheles/ Culex/ Aedes* larvae/ pupae in the MMF treated and control habitats (Type of habitat)

District..... PHC/CHC..... Village.....

Experiment No: ..... MMF: .....Treatment date: .....

Test species: .....Habitat water temperature: .....

Evaluation done by: .....Supervised by:.....

Post treatment Evaluation	Mean no. of 5 dips			Remarks
	Pupae	I & II Instar larvae	III & IV instar larvae	
Day 0				
Day 2				
Day 7				
Week 2				
Week 3				
Week 4*				

*if the observation is made beyond week 4, additional rows may be added

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 to section 5.1.2.2). The differences between the dosages can be compared by two-way ANOVA (refer to section 5.1.2.2). 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.

6.2.2 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 (cement tanks/tubs, drums/ barrels, etc.). For *Cx. quinquefasciatus,* trials are not undertaken in simulated condition. For *Anopheles* and *Aedes* spp., cement tanks/tubs, each having a capacity of 100 litres filled with 50 to 75 litres of potable water with different concentrations of MMF should be used. In each tank 100 to 200 first instar larvae of the target species should be released at weekly intervals until the completion of the experiment.

The tanks/tubs 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/tubs 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, obtained from Phase I laboratory evaluation or manufacturers label instructions (within the recommended range of dosages) should be applied to the tanks at the time of appearance of third instars using appropriate spray equipment. Each dosage 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 post-treatment counts (% reduction is calculated using the Mulla's formula) (Table 6.3). The minimum dosage causing the maximum reduction (>80%) for a longer duration should be selected as the optimum field application dosage for each habitat.

Table 6.3. Percentage reduction of larvae/ pupae in MMF treated habitats at the optimum application dosage

Experiment No: ..... MMF: ..... Treatment date: .....

Test species: .....Habitat water temperature: .....

Evaluation done by: ..... Supervised by:.....

Habitat	Day post-treatment	% Reduction		Remarks
		larvae	pupae	
	Day 0			
	Day 1			
	Day 2			
	Day 7			
	Week 2			
	Week 3			
	Week 4			

6.3 Phase III: Large-scale field evaluation (multicentric)

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

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

Objectives

- To confirm the efficacy of MMF against larvae/ pupae of target vector species in the selected 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 resident's acceptance.
- To observe the treatment effect on non-target organisms co-habiting with the target mosquito vectors.

6.3.1 Study Area

Two eco-epidemiologically comparable urban/rural areas should be selected for the trial, one for treatment and the other for control. All types of breeding habitats in the selected areas are surveyed to ascertain the breeding of the target vector species in order to decide the suitability of the localities for the trial. For this purpose, larval/pupal samples will be collected using an enamel dipper (300ml capacity) from different habitat types and allowed to emerge in the laboratory. The emerged adults will be identified to species using standard morphological identification keys. It is to be ensured that the areas selected for treatment and control should have adequate number of larval habitats of target vector (*Aedes*/*Anopheles*/*Culex*) species with comparable density. Species like *Cx. quinquefasciatus* breeds in polluted water with rich organic content, whereas Anopheles and Aedes species breed in clean water habitats. Separate trials should be carried out for the species that breeds in clean and polluted water habitats using the respective optimum field application dosage(s) determined in phase II trial.

Selection of larval habitats should be done carefully considering the residual activity of the formulations (as determined in phase II trials). Accordingly, the habitats which are likely to retain water for longer duration are selected. In other habitats with small volume of water, source reduction activities should be done. During trial period, many domestic/peri-domestic habitats are likely to get disturbed by frequent washing and other the activities of the residents. Considering all practical constraints, a minimum of 30 replicates of each type of major habitats (available in adequate numbers) should be selected for treatment and equal number of comparable habitats should be selected in a different area as controls. All the other larval habitats of the target vector species available in the trial area should be included for treatment and control. As in the case of small-scale field trials, each of the confined breeding habitats such as cesspits, borrow pits, cement tanks, wells can be considered as a discrete habitat or replicate. Habitats such as drains, irrigation canals, rice fields, ponds 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.

Post-treatment monitoring of the density of larvae and pupae in all the treated and control habitats should be assessed by taking fixed number of samples 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.

The vector species that breeds (*Cx. quinquefasciatus*) in perennial habitats, the entire trial will be repeated/replicated three times covering different seasons (summer, rainy and winter). In most of the epidemiological settings in India, profuse breeding of *Ae. aegypti* is observed during rainy season (June to September), the phase III trials against this species are mainly done during the rainy season. In other seasons adequate number of habitats may not be available for the trial.

Number of re-treatments may be restricted depending upon the residual activity of MMF and persistence of larval habitats. The habitats will be retreated at the intervals depending on the residual activity determined in phase II trial.

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 4.1.2.2 will be followed to confirm the residual efficacy and frequency of application.

7

Attractive Toxic Sugar Baits (ATSBs) – *Trial Version*

The current control measures against malaria vectors mainly rely on long-lasting insecticide nets (LLIN) and indoor residual spraying (IRS). For the control of vectors of dengue/chikungunya viruses, besides source reduction, chemical larviciding and thermal fogging activities are currently practiced. Emergence of insecticide resistance reduces the effectiveness of the current chemical control measures. This has necessitated the development of novel tools for use in integrated vector management (IVM). Attractive toxic sugar baits (also termed as Attractive targeted sugar baits) (ATSB) are one such new tool being developed and tested.

Attractive toxic sugar baits (ATSBs) use fruit or flower scent as an attractant, sugar solution as a feeding stimulant (bait) and chemical insecticide as a toxin to attract and kill the target vector mosquitoes and other biting flies. Female and male mosquitoes need plant derived sugar and carbohydrates to maintain energy for their survival. The sugar seeking behaviour offers an opportunity to leverage the sugar-feeding process with a bait containing a toxicant. ATSB solutions can be either sprayed on vegetation or suspended in bait stations to attract mosquitoes and kill them. Suppressing the vector populations has a beneficial impact on malaria and/or other vector-borne disease transmission.

This tool has been evaluated against anopheline and culicine vectors and also sand flies in many countries as a standalone vector control measure or as a supplementary measure along with LLINs. Although, efficacy trials with ATSB formulations are not being carried out currently in India, this new tool may assume importance as one of the vector control options for use in IVM programme in near future. Presently, no guidelines are available for evaluation of ATSB formulations. Hence, this guideline is developed to evaluate ATSB products in laboratory, small-scale and large-scale field conditions. This is only a trial version and as and when additional data/information relating to evaluation of ATSB are generated, the current version will be updated.

ATSB and ASB compositions: Attractive toxic sugar bait (ATSB) comprises of a bait attractive to the prevalent mosquito vector species of importance (usually with a locally available fruit or flower scent), a sugar solution as a feeding stimulant and a chemical insecticide (active ingredient) as an oral toxin to kill the target vector mosquitoes. Attractive sugar bait (ASB) is similar to ATSB but without toxin.

Different classes of oral toxins such as carbamates, pyrethroids, neonicotinoids, spinosyns, and borates are available for incorporating into ATSB. A wide range of active ingredients such as spinosad, pyriproxyfen, boric acid, eugenol, dinotefuron and microencapsulated garlic oil have been incorporated into ATSB and their efficacy has been demonstrated in various field trials.

As for other insecticide-based vector control interventions, the toxins used for ATSB formulations will be those that are approved by CIBRC considering the safety of the toxins to the non-target organisms including honeybees.

Before taking up the evaluation, material safety data sheet (MSDS) of the toxin in the ATSB formulation, preliminary data on its bio-efficacy (if available) and the technical specifications/ certificate of analysis (CoA) of the toxin should be obtained from the sponsor/manufacturer.

7.1 Phase I: Laboratory evaluation

Phase I evaluation is carried out in the laboratory to assess the efficacy and persistence of ATSB formulations incorporated with a candidate insecticide against laboratory reared target vector species. The ATSB formulation containing 6–7 concentrations of a candidate insecticide should be prepared and evaluated.

{Note: If a candidate insecticide is intended for use in ATSB formulation, the intrinsic (inherent) insecticidal activity, irritant or excito-repellent properties, the efficacy (the dosage that kill 50% (LC_{50}) and 99.9% ($LC_{99.9}$) of test individuals) and its persistence (residual activity) should be studied in laboratory following the procedures as described in section 2.1.}

Duration: 6 months

Objectives

- To assess the efficacy of the ATSB against target vector species and its persistence on sprayed plants or in soaked cotton pads.
- To establish dose-response relationship of the ATSB formulation against the target vector species.
- To determine the lethal concentration that kills 50% (LC_{50}) and 99.9% ($LC_{99.9}$) of the exposed vector population and to determine the dosages for Phase II trials.

7.1.1 Mosquito strains and colony

Laboratory bioassays with ATSB formulations are carried out using 3–5 days old, non-blood fed mosquitoes. Laboratory reared susceptible strains of the target vector species should be used for conducting laboratory experiments. Alternatively, F_1 progeny of field collected mosquitoes can be used. The target mosquito species are reared and maintained in the insectary under controlled temperature (27 ± 2°C) and relative humidity (80 ± 10%), with a photoperiod of 12 L:12 D.

7.1.2 Preparation of ASB solution utilizing locally available fruit juices or flower scent

The ASB solutions are prepared using locally available common fruit juices/fruits such as Mango (*Mangifera indica*), guava (*Psidium guajava*), banana (*Musas apientum*), honey/musk melon (*Cucum ismelo*), etc., and a blend of preservatives and slow-release substances. Fruit syrups such as date syrup which are reported to be effective can also be used.

7.1.3 Preparation of serial concentrations of ATSB with a candidate insecticide

The ATSB formulations are prepared by adding desired concentrations of active ingredient of toxins (candidate insecticides) to ASB.

Initial laboratory bioassays are to be carried out to determine the activity range of the candidate insecticide by exposing the adult mosquitoes to a wide range of 10–12 test concentrations. Based on adult mortality obtained in the wide range of concentrations, a narrow range of 6–7 concentrations causing mortalities between 10% and 95% at 24h or at 48h should be used for the laboratory bioassays carried out to determine LC_{50} and $LC_{99.9}$ values. Test concentrations should be

chosen in such a way that at least one concentration gives 100% and the other one gives ~50% mortality, two concentrations give ≥50% mortality, and at least two give between 5% and 50% mortality. Coloured food dye should be added to the ATSB and ASB solutions to determine the feeding status on ATSB/ASB.

If the sponsors/manufacturers provide dosages for Phase II testing or if the dosages against Indian vector species are already determined in the laboratory, determination of LC_{50} and $LC_{99.9}$ in Phase I may be skipped.

7.1.4 Laboratory bioassays with ATSB formulations

7.1.4.1 Efficacy of ATSBs on target vector mosquitoes

The efficacy of ATSB formulations incorporated with 6–7 concentrations of a candidate insecticide should be tested against target vector species in the laboratory. The ATSB and ASB will be provided to the vector mosquitoes on cotton wool pads saturated with 5 ml of the respective ATSB/ASB solutions. Cotton wool pads should be placed on the top of screened cups of 300 ml capacity (11 cm height × 9 cm diameter). Alternatively, laboratory bioassays can also be carried out using small mosquito cages (15 × 15 × 15 cm instead of using screened cups). Ten replicates, each with 10 (ten) (3-5 days old, non-blood-fed) mosquitoes (male: female ratio 1: 1) should be used for each treatment (ATSB) and control (ASB). Prior to the bioassay, it is to be ensured that the mosquitoes are starved (deprived of sugar meal) for at least 10-12h. Mosquitoes are left overnight (12 h) to feed on ATSB and ASB solutions and then scored in the morning as alive or dead and fed or unfed. In case of *Aedes sp*. experiment will be conducted during day time. Live mosquitoes should be transferred to clean paper cups with nylon net fastened to the rim by a rubber band, provided with a 10% sucrose cotton pad, and held under controlled temperature (27 ± 2°C) and relative humidity (80 ± 10%) to observe 48 h delayed mortality. Mosquitoes that fed or engorged with the coloured food dye marked bait solutions should be identified by observing the dye through the cuticle of the abdomen under a stereo microscope. The experiment should be repeated for three times on different days with different batches of mosquitoes using freshly prepared ATSB and ASB solutions.

7.1.4.2 Efficacy of ATSBs sprayed on plants

The ATSB solutions with 6–7 concentrations of a candidate insecticide should be tested against laboratory reared target vector species. For each test concentration, four screened cages (replicates) should be used. The size of the cage should be sufficiently large enough to accommodate a potted plant, (approximately 60 cm × 40 cm × 32 cm). This experiment should be performed in a laboratory maintained at a temperature of 27 ± 2°C and a relative humidity of 80 ± 10%. In each cage (replicate), one potted plant (available locally and used commonly as indoor/outdoor plant) should be kept. The flowers should be removed from the plant and then sprayed with 50 ml (adequate to cover the plant kept inside the cage) of respective concentration of ATSB solution and transferred to the respective labelled cages. Four separate cages containing 4 plants of the same species, should be sprayed only with ASB as controls. One hour after applying the solution, 3–5 days old, 200 mosquitoes (male: female ratio 1:1), starved at least for 10–12 h should be released in each cage. Mortality of the mosquitoes in treatments and control should be recorded after 48 h (72 h for late acting insecticides). The experiment should be repeated three times, using fresh ATSB /ASB solutions and different batches of mosquitoes.

7.1.5 Data Analysis

For determination of LC_{50} and $LC_{99.9}$ data from all replicates should be pooled. The LC_{50} and $LC_{99/9}$ are calculated from a log dosage-probit mortality regression line. Standard deviation or confidence intervals for the mean values of LC_{50} and $LC_{99.9}$ should be calculated and recorded in a format. The test series becomes valid if the standard deviation (or coefficient of variation) is less than 25% or if the confidence limits of LC_{50} and $LC_{99.9}$ values overlap (significant level at $P < 0.05$).

7.1.6 Determination of residual toxicity of ATSB in the laboratory

The residual toxicity of ATSB on plant surfaces can be studied initially in the laboratory using approximately 1.4 m^3 screened cages (0.92 m wide 1.68 m high 0.92 m depth), each containing a 12- liter pot (pot size: 30 cm diameter top; 24.2 cm diameter bottom; 30 cm depth) with a single non-flowering locally available plant (approximately 90 cm height and 60 cm wide). In each of the treatment cages, the plant should be sprayed with one of the test concentrations of ATSB (100 ml), enough to wet all surfaces of the plant thoroughly, but without runoff. In the control cage, the plant should be sprayed with 100 ml of ASB solution only. Once the spray is dry, 200 mosquitoes of the target vector species (sex ratio 1:1) should be released into each cage. Forty-eight hours (72 h for late acting insecticides) later, the dead mosquitoes in each cage and/or on the plant should be collected and their numbers recorded. The remaining live mosquitoes in the cages should be removed using a mouth/mechanical aspirator. Subsequently, on days 7, 14, and 21 post-treatments, 200 adult mosquitoes should be released into each of the treatment and control cages. The numbers of dead mosquitoes should be recorded after 48 h/72 h and the tests should be repeated for three times using freshly prepared ATSB and ASB solutions and different batches of mosquitoes.

7.2 Phase II - Small-scale field evaluation of ATSBs against anopheline and culicine vectors

Phase II trials are carried out against the natural population of target *Anopheles/Culex/Aedes* vector species or in simulated field conditions using laboratory reared target vector species. The application dosages of ATSB should be determined by multiplying the calculated $LC_{99.9}$ value determined in Phase I trial with a factor of 2 and above so as to obtain at least a range of 4–5 dosages for phase II trial. In case of indoor resting vectors, phase II trials should be carried out in experimental huts using ATSB bait stations. Against outdoor resting mosquito vectors, the ATSB formulations are sprayed on vegetation or suspended in ATSB bait stations in peri-domestic areas (outdoors) and evaluated.

Duration:12 months

Objectives

- To assess the efficacy and residual activity of ATSB formulations against natural population of the target vector species
- To determine the optimum application dosage of the ATSB formulation for Phase III evaluation.
- To study the impact of ATSB formulations on the behaviour of mosquitoes (deterrence, exophily, bait feeding, non-bait feeding, immediate and delayed mortality)

7.2.1 Phase II dosage determination trial in experimental huts

The experimental huts are specially designed for recording the entering and exiting behaviour of mosquitoes and for measuring response to ATSB formulations including mortality. Five to six experimental huts (depending on the number of treatment arms) should be used for the trial. The trial site should be located adjacent to a village/residential area and have adequate number of breeding habitats of target vector species to ensure high abundance of the target vector species.

In each of the experimental huts, an adult volunteer should be allowed to sleep under untreated bed nets from dusk to dawn during the entire trial period.

7.2.1.1 Design of experimental huts

The design and dimensions of the huts should resemble almost to those of the village huts. The huts are constructed of bricks with cement plastering outside and thatched/corrugated iron roof. Inner walls of the huts are plastered with mud. The experimental hut consists of a single room

with four windows, two windows on the front door side and one on each side of the room. The huts are built on plinths and surrounded by water filled moats to prevent entry of scavenging ants and flooding water during the rainy season. The design and construction details of experimental huts are described in the section 2.2.1.1.

7.2.1.2 Acclimatization and suitability of huts to ATSB evaluation

Prior to the hut trial, assessment of suitability of the huts is essential to ensure that the huts are comparable in their attractiveness to the target vector species. And to ensure that the huts are tight enough and free from contamination with insecticides and mosquito scavengers such as ants (Refer to section 2.2.1.2).

7.2.1.3 Ethical clearance

Approval of the Institutional Human Ethics Committee should be obtained to involve human volunteers in the study. Signed written informed consent forms should be obtained from the trial participants and they should be offered free medical services during the trial up to three weeks after the end of participation in the trial.

7.2.1.4 Experimental arms

In Phase II evaluation in experimental huts, 4–5 concentrations of ATSB formulation can be tested. For example, in a six-arm trial, the following five treatments (T1-T5) and one control (T6) are evaluated and compared.

T1: ATSB with $LC_{99.9}$ x2 of a.i of insecticide + untreated nets
T2: ATSB with $LC_{99.9}$ x4 of a.i of insecticide + untreated nets
T3: ATSB with $LC_{99.9}$ x6 of a.i of insecticide + untreated nets
T4: ATSB with $LC_{99.9}$ x8 of a.i of insecticide + untreated nets
T5: ATSB with $LC_{99.9}$ x10 of a.i of insecticide + untreated nets
T6: ASB Control (without toxin) + untreated nets

7.2.1.5 Preparation of ATSB & ASB formulations and bait stations

The ASB solutions should be prepared with the same components/ingredients used in phase I trial. The test formulations of ATSB are prepared by incorporating five concentrations of a.i of the candidate insecticide with the ASB solutions. Bait stations are constructed from a plastic soft drink bottle (1.5 l), in which a 2 cm hole is made at two-third height of the bottle as shown in Figure 7.1.

Figure 7.1: Construction of bait stations using locally available methods (Source: Qualls et al., 2015)

Cotton wick is inserted through the hole so that both the ends of the wick reach the bottom of the bottle, one inside and one outside. The bottles are then inserted into large, light-coloured cotton flannel socks that had been thoroughly washed with water and dried. Subsequently, the socks are soaked in either ASB or ATSB solutions. The bottles are then filled with 0.9 l of respective

concentrations of ATSB or ASB solution allowing for continuous seeping of the solution from the bottle through the wick so as to avoid complete drying of the external flannel coat.

{Note: When the bait stations are installed outdoors, they are protected from rain/sunlight with 60cm umbrella shaped plastic cover (Figure. 7.1)}

7.2.1.6 Pre-treatment assessment and sugar feeding status

Prior to running the full experimental hut trial, 12-night pilot trials should be carried out for a period of one month to observe mosquito bait feeding behaviour and to make adjustments in the study protocol. In the pilot trials, food dye marked ASB stations (without toxin) should be installed as per the experimental hut trial setup described below. The investigator should sleep in the huts throughout the pilot trials for making observations and necessary improvements to the design. Every day morning, mosquito collections should be carried out in the room and verandah trap in all the huts. The collected mosquitoes should be sorted gender-wise, identified, counted and their sugar feeding status should be determined by subjecting mosquito samples to anthrone test (Schlein & Jacobson 2019, Sissoko et al. 2019) or by visual demonstration (Kline et al 2018, refer section: 7.3.1.4.1). In control huts, optimal/sufficient number of samples of target vector species should be collected to ensure an adequate power to detect statistical significance between the treatments and control.

7.2.1.7 Dosage determination trial in the experimental huts

For each treatment (dosage), one experimental hut should be allocated. In each hut, four ATSB bait stations treated with one of the five concentrations of candidate insecticide should be hung from the ceiling at each corner of the untreated bed net (approximately 1.5 m above the ground level). Additionally, ATSB bait stations should also be set near each of the window with one bait station in the middle and two on either side of windows. Cotton pads soaked with 10% sucrose solution should be placed inside the window traps as well as veranda traps for those mosquitoes that exit/leave the huts without feeding on ATSB. In the control hut, ASB bait stations should be set as per the arrangement of ATSB bait stations in treatment huts.

The experimental hut trial duration depends upon the number of concentrations (treatment arms) used (32 nights for 6 arm trial). One adult male volunteer should be allowed to sleep in each hut under untreated bed net from 1900 to 0600 h. The ATSB bait stations in the treatment arms and ASB bait stations in control should be rotated on weekly basis using a Latin square design so that each treatment is tested with each volunteer and hut an equal number of times (Table 7.1). The ATSB and ASB bait stations are to be replaced weekly/fortnightly based on their residual activity.

Prior to rotating the bait stations, mosquito entrance should be blocked every sixth morning and live mosquitoes in the room should be given an additional 24 h to feed or exit the room before collection. Every seventh night, when the bait stations are rotated, live mosquitoes in the room should be removed and the rooms cleaned.

The bait stations with ATSB or ASB solutions should be set in the respective experimental huts allocated. Each day morning at 0600 h, mosquito collection should be carried out by two trained insect collectors using mouth/mechanical aspirators. Fifteen minutes to be spent per room and veranda. The dead and live mosquitoes should be collected from the verandah trap and only dead mosquitoes from the floor of the room. The live mosquitoes in the room should be left to exit from the rooms deliberately. The collected mosquitoes should be scored as live or dead or bait fed or non-bait fed and identified to species level. The live mosquitoes (about 10 mosquitoes per cup) from the veranda trap should be placed in clean paper cups (300 ml capacity) and provided with 10% sucrose cotton pads and held under $27 \pm 2°C$ with a relative humidity of $80\% \pm 10\%$ for observing delayed mortality.

Table 7.1. Latin Square ROTATION scheme for treatment arms (ATSB concentrations) and sleepers (6 arms)

		Rotation of treatment arms between the huts						Rotation of volunteer between the huts					
Week	**Day**	**H1**	**H2**	**H3**	**H4**	**H5**	**H6**	**H1**	**H2**	**H3**	**H4**	**H5**	**H6**
1	1.	T1	T2	T3	T4	T5	T6	A	B	C	D	E	F
	2.	T1	T2	T3	T4	T5	T6	B	C	D	E	F	A
	3.	T1	T2	T3	T4	T5	T6	C	D	E	F	A	B
	4.	T1	T2	T3	T4	T5	T6	D	E	F	A	B	C
	5.	T1	T2	T3	T4	T5	T6	E	F	A	B	C	D
	6.	T1	T2	T3	T4	T5	T6	F	A	B	C	D	E
	7.	Ventilating, cleansing and washing of hut						No volunteers sleeping inside the hut					
2	8.	T2	T3	T4	T5	T6	T1	A	B	C	D	E	F
	9.	T2	T3	T4	T5	T6	T1	B	C	D	E	F	A
	10.	T2	T3	T4	T5	T6	T1	C	D	E	F	A	B
	11.	T2	T3	T4	T5	T6	T1	D	E	F	A	B	C
	12.	T2	T3	T4	T5	T6	T1	E	F	A	B	C	D
	13.	T2	T3	T4	T5	T6	T1	F	A	B	C	D	E
	14.	Ventilating, cleansing and washing of hut						No volunteers sleeping inside the hut					
3	15.	T3	T4	T5	T6	T1	T2	A	B	C	D	E	F
	16.	T3	T4	T5	T6	T1	T2	B	C	D	E	F	A
	17.	T3	T4	T5	T6	T1	T2	C	D	E	F	A	B
	18.	T3	T4	T5	T6	T1	T2	D	E	F	A	B	C
	19.	T3	T4	T5	T6	T1	T2	E	F	A	B	C	D
	20.	T3	T4	T5	T6	T1	T2	F	A	B	C	D	E
	21.	Ventilating, cleansing and washing of hut						No volunteers sleeping inside the hut					
4	22.	T4	T5	T6	T1	T2	T3	A	B	C	D	E	F
	23.	T4	T5	T6	T1	T2	T3	B	C	D	E	F	A
	24.	T4	T5	T6	T1	T2	T3	C	D	E	F	A	B
	25.	T4	T5	T6	T1	T2	T3	D	E	F	A	B	C
	26.	T4	T5	T6	T1	T2	T3	E	F	A	B	C	D
	27.	T4	T5	T6	T1	T2	T3	F	A	B	C	D	E
	28.	Ventilating, cleansing and washing of hut						No volunteers sleeping inside the hut					
5	29.	T5	T6	T1	T2	T3	T4	A	B	C	D	E	F
	30.	T5	T6	T1	T2	T3	T4	B	C	D	E	F	A
	31.	T5	T6	T1	T2	T3	T4	C	D	E	F	A	B
	32.	T5	T6	T1	T2	T3	T4	D	E	F	A	B	C
	33.	T5	T6	T1	T2	T3	T4	E	F	A	B	C	D
	34.	T5	T6	T1	T2	T3	T4	F	A	B	C	D	E
	35.	Ventilating, cleansing and washing of hut						No volunteers sleeping inside the hut					
6	36.	T6	T1	T2	T3	T4	T5	A	B	C	D	E	F
	37.	T6	T1	T2	T3	T4	T5	B	C	D	E	F	A
	38.	T6	T1	T2	T3	T4	T5	C	D	E	F	A	B
	39.	T6	T1	T2	T3	T4	T5	D	E	F	A	B	C
	40.	T6	T1	T2	T3	T4	T5	E	F	A	B	C	D
	41.	T6	T1	T2	T3	T4	T5	F	A	B	C	D	E
	42.	Ventilating, cleansing and washing of hut						No volunteers sleeping inside the hut					

ATSB Concentrations/ASB	Volunteer (Sleeper)
ArmT1, ATSB	A
ArmT2, ATSB	B
ArmT3, ATSB	C
ArmT4, ATSB	D
ArmT5, ATSB	E
ArmT6, ASB	F

7.2.1.8 Indicators to be measured

Deterrence: the proportional reduction in hut entry relative to entry in the control hut

Exit rate: the proportion of mosquitoes found in the verandah trap

ATSB-feeding: proportion of mosquitoes with food dye found in their abdomen (by examining under a microscope) or by anthrone test

Mortality: This includes the proportion of mosquitoes found dead in the morning collection and 72 hour delayed mortality of the total mosquitoes entered the hut (the proportion of mosquitoes found dead 24, 48 and 72 h after collection).

7.2.1.9 Data analysis

Data analysis should be carried out using appropriate statistical method/methods. The analysis of the phase II experimental hut data should be done using logistical regression for proportional data (proportion dead, marking with dye of the total, marking with dye of the dead, and dying of the dyed) and adjusted for the effects of individual sleepers and huts.

7.2.2 Phase II evaluation of ATSB in village huts

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

7.2.3 Phase II evaluation of ATSB stations set at residential back yards (outdoors)

The trials can be conducted on natural populations of mosquitoes in the residential areas, setting ATSB bait stations during peak transmission/mosquito season. Six residential backyards, each with approximately 5000 m^2 (72 m $\times$ 72 m) should be selected for the trial (five for treatments and one for control). Selected sites should be comparable in terms of target vector density and vegetation type and cover and separated at least by 2 km away from each other.

Prior to installation of bait stations, the density of target vector species and their sugar feeding status should be monitored using appropriate trapping/sampling device (CDC miniature light traps/UV light traps for *Anopheles* and *Culex* vectors and BG sentinel for *Aedes* species) for a fortnight period and the mosquito collections should be carried out twice a week. At each yard, two traps should be placed in the front yard and another two traps placed in the backyard. In each of the selected yards, one of the five concentrations of ATSB bait stations should be tested and the sixth yard should be used as control, tested with only ASB (without toxin) bait stations. The treatments and control arms should be allocated randomly. In each of the treatment yards, sixteen bait stations should be (including front and backyard) set along the boundary of the residence at 25 m intervals.

In each yard, the number of landing mosquitoes should be recorded at 72 h post- installation of the bait stations by a human volunteer. The human volunteer standing/sitting in each corner of each yard for half an hour during dusk will collect the landing mosquitoes on his exposed forearms/legs. Subsequently, collection of adult mosquitoes should be carried out in each yard using the same collection/trapping device used during pre-treatment. All the collected mosquitoes

during the pre- and post-installation of ATSB stations are to be identified, counted, and recorded. Monitoring of post-treatment density should be carried out on day 7, 14, 21 and beyond at weekly intervals, if needed, to measure the persistence of the ATSB application. The experiment should be repeated three times.

Data analysis: The difference in the mean man landing counts and trap catches between different concentrations from the field study will be tested using analysis of variance (ANOVA), with means separation using the least significance difference (LSD) test and a probability level of 0.05.

7.2.4 Phase II evaluation of ATSB formulations sprayed on vegetation against natural vector population

The trial (spraying on vegetation) should be carried out against the vector species that shows peak activity during post-monsoon season. The efficacy of ATSB formulation sprayed on vegetation can be evaluated in residential backyards. Six sites (number of sites depends upon the number of treatment arms) should be selected based on visually observed similarity in vegetation cover infested with target vector species. The vegetation present including landscaping vegetation in the selected sites should be recorded. The selected sites should be separated from each other (at least by 1 km) considering the flight range of the target vector species. In each site, one of the five concentrations of ATSB solutions should be applied. In the sixth site (control), ASB should be applied. Pre-treatment density of the target vector species in the treatment and control sites should be monitored for a week at least on two occasions. In each site, three traps (appropriate to the target vector species) should be set on each day of sampling. The ATSB solution is applied with handheld compression sprayer on perimeter foliage from the ground to 1 m high. The application should be made to patches of vegetation from top to bottom and left and right consistently, in a sweeping pattern. Monitoring should be done on day 7, 14, and 21 post-ATSB application to measure the persistence. Three traps should be set in each of the selected sites. In addition, oviposition traps (for *Aedes sp.*) can also be used to monitor the efficacy of the ATSB application during pre- and post-treatment. Both the ATSB traps and oviposition traps should be placed in vegetative areas of the residential sites and should be separated by at least 10 m.

Data analysis: The mean number of adults and eggs collected during pre- and post-ATSB application in residential backyards should be analyzed using two-way ANOVA. Percent reduction in adult density and egg counts between the treatments and control should be determined by using the formula:

$$[100 - \{(\text{pre-treatment control numbers}/\text{pre-treatment experimental numbers}) \times (\text{post treatment control numbers}/\text{post-treatment experimental numbers})\} \times 100]$$

7.2.5 Phase II evaluation of ATSB formulations sprayed on vegetation under semi-field conditions

The efficacy and residual activity of ATSB formulations applied to vegetation can also be evaluated under relatively controlled and comparable settings. In areas where adequate number of isolated and comparable outdoor sites with similar type of vegetation infested with target mosquito species are not available, separate trial should be carried out under semi-field conditions.

Duration: 6 months

The trial should be conducted under semi-field-controlled conditions using laboratory reared *Anopheles*/*Culex*/*Aedes* vectors during post-monsoon season. The ATSB formulations should be tested at 4–5 concentrations. The dosages for application in Phase II field trial should be in the range of 2 to 10 fold of the $LC_{99.9}$ determined in the Phase I trial.

7.2.5.1 Mosquito strains and colony

Target vector species can be reared and maintained in the insectary at $27 \pm 2°C$ and $80 \pm 10\%$ relative humidity in a 12:12 light:dark photoperiod using the methods described in Phase I trial (Section 6.1.1). Three to five days old, non-blood fed mosquitoes should be used for the trial.

7.2.5.2 Evaluation under semi-field conditions

Trial should be conducted in six large outdoor screened enclosures 72–90 m^3, 5 for treatments and one for control. The interior of each enclosure should be provided with similar numbers of locally available potted plants. Fourteen plants (approximately 1.5 m tall, 0.75–0.9 m wide, each in 20 liters pots) should be arranged in a circle in the center of each enclosure. One of the five concentrations of ATSB (one litre) should be evenly applied using a 10 l hand compression sprayer to all plant surfaces (foliage and stems) but without runoff. The sixth enclosure (control) should be sprayed with ASB solution. The wet plants should be allowed to dry (60–120 minutes). Later, 500 adult mosquitoes (sex ratio 1:1; 3–5 days old, starved at least for 10–12 h) should be released into each enclosure. At 48 h/72 h post-treatment, one human volunteer standing/sitting in the center of each enclosure should record the numbers of mosquitoes that land/probe on both forearms/legs during a series of three min long observation periods, five min apart for half an hour period. Following these observations, collection of adult mosquitoes in each enclosure should be carried out for 12 h using appropriate trapping device (depending upon the target vector) described in section 7.2.2.

7.2.5.3 Determination of residual activity

The duration of ATSB toxicity on plant surfaces should be tested by releasing 500 adult mosquitoes of the same species (3–5 days old, non-blood-fed), but different batches into each of the enclosures on day 7, 14 and 21 post treatment. The number of mosquitoes landing on a human and the number of mosquitoes captured using the same trapping devices should be recorded. These tests should be repeated three times using freshly prepared ATSB and ASB and different batches of the target vector species. The same human volunteer should be engaged in all the tests.

7.2.5.4 Data analysis

Mosquito responses in each test should be analysed using multiple variance (ANOVA) procedures. The data on man landing counts and trap catches should be transformed to log10 (n + 1) before analysis. Mean values in each test should be compared using least significant difference (LSD) test.

7.3 Phase III evaluation of ATSBs against *Anopheles / Culex* vectors (multi-centric)

The efficacy of ATSB that showed desired activity in experimental hut or small-scale outdoor field trials (Phase II) should be evaluated in large-scale (at village level) against the target vector mosquitoes at least in three eco-epidemiological settings (multi-centric) covering peak mosquito/ transmission seasons. The Phase III trials are carried out at village level, selecting comparable villages in terms of population, housing structure, mosquito species and density, topography and disease prevalence/incidence.

Duration: 12 months

Objectives

- To assess the impact of attractive toxic sugar bait formulations (ATSBs) on longevity, density and infectivity rate of the target vectors when applied at the optimum field application dosage on vegetation or used in bait stations in the residential areas.
- To study the impact of ATSB on disease incidence/ prevalence
- To assess community acceptability of the ATSBs formulations.

7.3.1 Phase III evaluation of ATSB stations set outdoors against exophilic *Anopheles/ Culex/Aedes* vectors

7.3.1.1 Study Area

The Phase III trials are generally designed as cluster randomized trials and the unit of intervention under this phase is the village. The villages to be selected for the evaluation should be at least 2 km

away from each other to avoid infiltration of mosquitoes in to the treatment area from the control area or vice versa. The effect of active ingredient (insecticide) used in the ATSB formulations is to reduce the longevity, density and infectivity rate of the target vectors. Three villages, each with a population of approximately 1000 should be selected for treatment (ATSB). For comparison, three villages with comparable population size should be selected as control.

7.3.1.2 Collection of base line data

The pre-treatment density of target vector species should be monitored in all six villages selected fortnightly for a period of three months using appropriate sampling methods.

(***Note:*** *Appropriate trapping devices/collection methods should be selected based on the target vector species to be tested).*

7.3.1.2.1 CDC traps/BG sentinel traps

In each village, ten CDC UV light traps/CDC miniature light traps (*Anopheles/Culex vectors*) should be set up in ten outdoors sites at least 10 m apart. The traps should be set approximately 3–5 m away from the households and run for ~12 h (from dusk to dawn). This should be conducted in each village at fortnightly interval. For monitoring diurnally active *Aedes* vectors, BG sentinel traps with lure should be used at ten sites in each village. The traps should be set at dawn and removed at dusk.

7.3.1.2.2. Hand catches of indoor resting mosquitoes

Hand catches of indoor resting mosquitoes should be done using either mechanical aspirator/ prokopack aspirator in ten houses (10 minutes per house) per village at fortnightly intervals to measure the target vector density, which is expressed as the number per man-hour (man-hour density, MHD).

7.3.1.2.3 Human landing catches (HLC)

Human landing catches may be carried out after obtaining necessary clearance from institutional human ethics committee. HLCs should be carried out both indoors and outdoors, each from two sites. The volunteers are allowed to sleep exposing their legs and the landing mosquitoes are collected using oral aspirators and recorded. The distance between the outdoor volunteers is at least 10 m and indoor volunteers are located in separate houses. Both indoor and outdoor volunteer locations are interchanged every 2 h to eliminate positional bias. HLC indoors (two sites) and outdoors (two sites) should be done fortnightly in each study village.

7.3.1.3 Bait station construction

Refer to section 7.2.1.5.

7.3.1.4 Treatment with ATSB

The efficacy of ATSB formulation at the selected optimum field application dosage (as determined in phase II experimental hut or small-scale outdoor field trial) should be tested in Phase III trial. ATSB bait stations are hung outdoors (2 per house on the exterior walls) in treatment villages prior to transmission season and are left until the end of the transmission period. The ATSB bait stations should be replaced with the respective baits at the frequency determined in phase II trial.

7.3.1.5 Monitoring the post-treatment density of target vector population

The target vector populations in all experimental and control villages should be sampled fortnightly to monitor the density as described in section 7.3.1 during the entire period of experiment. Processing of pre- and post-treatment collections should include mosquito identification, blood feeding status, determination of parity and vector infection rate.

The abdominal condition of all the vector mosquitoes collected from experimental and control villages is examined and the blood feeding status recorded.

7.3.1.6 Determination of parity

Random samples of 100 unfed females collected by each trapping method, are analyzed for parous and nulliparous conditions following tracheolor skein method (coiled-nulliparous; uncoiled-parous). In situations where, adequate number of unfed mosquitos are not collected, ovariolar dilatation method can be applied for parity assessment using the mosquitoes of other gonotrophic conditions.

7.3.1.7 Determination of vector infection rate

All the female mosquitoes collected will be processed for determination of vector infection/ infectivity rates following appropriate techniques, dissection for examination of midgut and salivary gland or ELISA/PCR.

7.3.1.8 Determination of entomological inoculation rate (applicable only to malaria vectors)

Entomological inoculation rate (EIR) is the number of infective bites per person per night. It is estimated from the product of sporozoite rate multiplied by the human landing density.

7.3.1.9 Community acceptability

Acceptability of ATSB treatment depends upon the benefits perceived by the community and the degree of inconvenience and any side-effects caused by the treatment. Any inconvenience caused by the treatment and perceived risks may lead to refusal. In order to assess the acceptability of ATSB formulations, baseline data should be collected from a random sample of households in the treatment villages during the pre-treatment period. Acceptability of ATSB formulations should be assessed on two-week post-treatment and thereafter every month until completion of the trial. A qualified social scientist should be engaged to develop a culturally sensitive questionnaire, that should be pre-tested before use. Focus group discussions may be conducted to obtain qualitative information.

7.3.1.10 Data analysis

For the Phase III evaluation, the primary unit of replication and analysis is the village. Therefore, an appropriate statistical method that adjust the variation existing between villages should be used for analysis before estimating the effect of the ATSB application comparing treated villages with the control villages. Multivariate analysis is therefore the preferred approach since it takes in to account such variations. Data on proportions (e.g., parous rates, infection rates) are 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 percent reduction in the mosquito populations is calculated by determining the pre-treatment populations compared to post-treatment populations [100 – [(pre-treatment control village numbers/ pre-treatment experimental village numbers) × (post-treatment control village numbers/ post-treatment experimental village numbers)] × 100].

Data on disease incidence obtained from the state/district Health Department may be compared between the experimental and control villages to assess the impact of ATSB.

7.3.2 Phase III evaluation of ATSB stations set inside human dwellings against endophilic *Anopheles/Culex/Aedes* vectors

7.3.2.1 Selection of study sites

Refer to section 7.3.1.1.

7.3.2.2 Collection of base line data

Pre-treatment population densities should be monitored in all six villages selected at fortnightly interval for a period of three months. Pyrethrum spray catches (PSC) or hand hatches of indoor

resting mosquitoes (10 minutes per house) using mechanical aspirator/prokopack aspirator should be done in six to ten houses per village to monitor the indoor-resting density of male and female mosquitoes of *Anopheles/Culex/Aedes* vectors in all the six study villages. For monitoring the density of *Aedes* species, BG sentinel traps without lure should be used in ten houses (preferably in bed rooms) in each village. The traps should be set at dawn and removed the next day at dawn.

7.3.2.3 Construction of ATSB station

Refer to section 7.2.1.5

7.3.2.4 Treatment with ATSB

Following the pre-treatment evaluation, food dye-marked ATSB stations are hung in all the bedrooms (one per bedroom) in the treatment villages.

Mosquito populations are then monitored in both treatment and control villages fortnightly atleast, until the end of transmission season by randomly selecting ten houses per sampling period. All rooms within the designated ten houses are sampled using PSC or hand catch method to evaluate the effect of the ATSB indoor bait stations on mosquito populations. Bait stations are replaced with new ones at an interval as determined in Phase II trial.

All mosquitoes collected are sorted to males and females and identified to species following standard morphological keys. Post-treatment mosquito collections in treatment villages are visually inspected with a dissection microscope for the presence of food dye to determine the feeding rate on ATSB (post-treatment; treatment villages only) stations. In the absence of food dye in the abdomen of the mosquitoes collected in the treatment sites during post-treatment, the dissected guts are screened for their sugar-feeding status by anthrone testing. The female mosquitoes collected from both treatment and control villages are also assessed for their blood feeding status. Additional dissections are performed for parous and nulliparous condition using tracheolar skein/dilatation method and for determining infection rates.

7.3.2.5 Data analysis

Refer to section 7.3.1.10

7.3.3 Phase III evaluation of ATSB formulations sprayed on vegetation against outdoor resting *Anopheles/Culex/Aedes* vectors

7.3.3.1 Selection of study sites

Phase III evaluation of ATSB formulations against outdoor resting mosquito vectors should be carried out in the residential backyards (outdoors) covering the transmission season. Six comparable outdoor sites each with a human population of approximately 1000 should be selected based on visually observed similarity in preferably shrub vegetation cover. The selected residential outdoor sites should have adequate number of larval habitats of the target vector species and the density of the target vector species to be tested should be comparable. The major landscaping vegetation and the secondary vegetation dominating the study sites should be recorded. Three sites should be allocated to treatment randomly and another three sites to control. The control sites should be at least 2 km away from the selected treatment sites to avoid infiltration of mosquitoes in to the treatment area.

7.3.3.2 Monitoring of pre-treatment density

In each study village, pre-treatment monitoring of mosquito density should be carried out at fortnightly interval for a period of three months using appropriate sampling method. Six CDC miniature light traps/UV CDC light taps (without lure) should be used in each of the selected village for monitoring the density of *Anopheles/Culex/Aedes* vectors. Traps should be suspended from bamboo/wooden tripods ~1 m from the ground in fixed locations and are turned on at dusk and collected in dawn.

For *Aedes* species, six BG sentinel traps with lure should be used in each of the selected villages for monitoring the density of *Aedes* vector. The traps should be set at dawn and removed at dusk.

In addition, hand catches or pyrethrum spray sheet collections of indoor resting mosquitoes will be carried out as described in sections 7.3.1.2.2. Human landing collections will also be carried out in treatment and control villages (refer to section 7.3.1.2.3).

7.3.3.3 Application of ATSB formulations

The optimum application dosage of ATSB formulation (as determined in phase II trial) should be sprayed in the treatment sites using a standard 16-liter back-pack sprayer in aliquots of ~80 ml on 1 m^2 spots at distances of ~3 m on the vegetation at the residential backyards.

7.3.3.4 Monitoring of post-treatment density

Post-monitoring of mosquito density should be done at fortnighly interval until two weeks after the end of transmission season. For *Aedes* sp. BG sentinel traps should be used. In addition, oviposition traps should be used to monitor the efficacy of the ATSB application. Both BG and oviposition traps should be placed in vegetative areas of the residential sites and are separated by 10 m. For post-monitoring the density of *Anopheles*/*Culex* vector, CDC miniature light traps (without lure) should be used in each of the selected village at fortnighly interval.

In addition, hand catches or pyrethrum spray sheet collections of indoor resting mosquitoes will be carried out as described in sections 7.3.1.2.2. Human landing collections will also be carried out in treatment and control villages (refer to section 7.3.1.2.3).

7.3.3.5 Testing for sugar feeding status and age grading of mosquitoes

Collected mosquitoes should be sorted to males to females and identified to species. The parity status (parous or nulliparous) will be assessed using tracheolar or dilatation method (refer to section 7.3.2.4). The blood feeding status and vector infection/infectivity rates are determined as mentioned in section 7.3.1.7. Mosquitoes collected from treated sites are checked for food dye marker under a dissection microscope.

7.3.3.6 Data analysis

Refer to section 7.3.1.10

8

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9

Annexures

Annexure 1

Guidelines for development of information sheet and consent form to involve human participants

(National Ethical Guidelines for Biomedical and Health Research involving Human Participants, 2017)

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. Generally, incentives are not encouraged. However, reimbursements for expenses incurred and loss of wages 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. Mention (wherever applicable) that the participant is insured by the funding agency /sponsor to meet financial compensation in case of unforeseeable event of death or disability arising out of (or attributable) to the study.

18. 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 ethics committee], whose task is to make sure that research participants are protected from harm. If you wish to find out more about the Local Ethics 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: _____/_____/_____ (dd/mm/yyyy)

*For children between 7 and 11 years of age, oral assent must be obtained in the presence of parent/LAR; for children between 12 and 18 years of age, written assent must be obtained; for children less than 7 years of age, parental consent is sufficient - please refer to ICMR guidelines 2017.

Annexure 2

Certificate of Consent of volunteers to sleep in experimental huts

(This is an integral part of the information sheet and not a stand-alone document)

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate as a sleeper in this study and understand that I have the right to withdraw from the study at any time without in any way affecting my medical care. I am told that the supervisor of the research team along with the village leader or his representative would make some unexpected visits in the night to ensure adherence of study procedures and might speak to me as a volunteer participant. I also understand that the Principal Investigator of the study can exclude me from the study if I do not adhere to the study procedures as described in the information sheet. I have been provided with a copy of this consent form.

Print Name of Volunteer (net user) Date and Signature of Volunteer (net user)

_____ _____/_____/_____ (dd/mm/yyyy)

If illiterate

I have witnessed the accurate reading of the consent form to the participant, and the individual had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print Name of Independent Literate Witness Date and Signature of Witness

(if possible, this person should be selected by

the participant and should have no connection

to the research team)

_____ _____/_____/_____ (dd/mm/yyyy)

I have accurately read or witnessed the accurate reading of the consent form to the participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print Name of Researcher Date and Signature of Researcher

_____ _____/_____/_____ (dd/mm/yyyy)

Name of Volunteer (net user) Date and Signature of Volunteer (net user)_____ _____/_____/_____ (dd/mm/yyyy)

Annexure 3

Certificate of Consent of volunteers to be human baits for mosquito landing collections

(This is an integral part of the information sheet and not a stand-alone document)

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent to take part voluntarily in this study as a human bait for mosquito landing collection and understand that I have also the right to refuse to be the human bait. I also understand that the Principal Investigator of the study can exclude me from the study with my consent. I have been provided with a copy of this consent form.

Print Name of patient/Villager

Date and Signature of patient/Villager

_____

_____/_____/_____ (dd/mm/yyyy)

If illiterate

I have witnessed the accurate reading of the consent form to the participant, and the individual had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print Name of Independent Literate Witness

(if possible, this person should be selected by

the participant and should have no connection

to the research team)

Date and Signature of Witness

_____

_____/_____/_____ (dd/mm/yyyy)

I have accurately read or witnessed the accurate reading of the consent form to the participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print Name of Researcher

Date and Signature of Researcher

_____

_____/_____/_____ (dd/mm/yyyy)

Name of Patient/Villager

Date and Signature of patient/Villager

_____

_____/_____/_____ (dd/mm/yyyy)

Annexure 4

Certificate of Consent for giving finger prick blood sample

(This is an integral part of the information sheet and not a stand-alone document)

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent to give my finger prick blood for this study and understand that I have the right to refuse to give my finger prick blood also. I also understand that the Principal Investigator of the study can exclude me from the study if I do not adhere to the study procedures as described in the information sheet. I have been provided with a copy of this consent form.

Print Name of patient/Villager

_____

Date and Signature of patient/Villager

_____/_____/_____ (dd/mm/yyyy)

If illiterate

I have witnessed the accurate reading of the consent form to the participant, and the individual had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print Name of Independent Literate Witness

(if possible, this person should be selected by

the participant and should have no connection

to the research team)

_____

Date and Signature of Witness

_____/_____/_____ (dd/mm/yyyy)

I have accurately read or witnessed the accurate reading of the consent form to the participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print Name of Researcher

_____

Date and Signature of Researcher

_____/_____/_____ (dd/mm/yyyy)

Name of Patient/Villager
Villager_____

Date and Signature of patient/
_____/_____/_____ (dd/mm/yyyy)

Annexure 5

Verbal/oral assent form for children between 7 and 12 years

(This is an integral part of the information sheet and not a stand-alone document)

{For children between 7 and 12 years, verbal/oral assent must be obtained in the presence of the parents/ legally acceptable/authorized representative (LAR) and should be recorded – ICMR Guidelines, 2017}

I have read the details given in the information sheet or it has been read to me, and fully understood the details of the study. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I have also been explained by the principal investigator or his duly authorized representative the full details, and having fully satisfied myself with the explanation given, of my own desire with full sense / awareness, give my consent for/ my son/ my daughter to give finger prick blood sample for screening for infection due to malaria. I hereby confirm that oral/verbal consent of my son/daughter has been taken by the Investigator after explaining the details of the study to him/her. Also, I give my consent for treating my child with the anti-malaria drugs for clearing infection, if he/she is found infected.

I am also fully aware of my right to withdraw my ward from the study, and in case should I stop my child participating, that my ward will be given treatment for the disease or will be referred for appropriate treatment.

Given this day.....in the month of .....in the year.....

Print Name of the Child: .....

Date & Signature of the Parent/LAR: .....

Date & Signature of the Witness

1. Address

.....

.....

2. Address

.....

.....

Print Name of Researcher :

Date & Signature of the Researcher

Annexure 6

Written assent form for participating children between 12 and 18 years

(This is an integral part of the information sheet and not a stand-alone document)

{For children between 12 and 18 years, written assent must be obtained. This assent form also has to be signed by the parents/legally acceptable/authorized representative (LAR).Adolescents may have the capacity to give consent like adults. However, as they have not attained the legal age to provide consent, it is termed as assent and the consent of the parents/LAR should be obtained – ICMR Guidelines, 2017}.

I have read the information given in the information sheet or it has been read to me, and fully understood the details of the study. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I have also been explained by the principal investigator or his duly authorized representative the full details, and having fully satisfied myself with the explanation given, of my own desire with full sense / awareness, give my consent for/my son/ my daughter to give finger prick blood sample for screening for infection due to malaria. Also, I give my consent for treating my child with the anti-malaria drugs for clearing infection, if he/she is found infected.

I am also fully aware of my right to withdraw my ward from the study, and in case should I stop my child participating, that my ward will be given treatment for the disease or will be referred for appropriate treatment.

Given this day.....in the month of .....in the year.....

Signature of the child

Signed in my presence

Signature of the parent

Signature of the witness

1. Address

.....

.....

.....

.....

2. Address

.....

.....

Name of the Researcher

Signature of the Researcher

Annexure 7

Assessment of adverse effects, acceptability by householders and collateral benefits of indoor residual spraying

Date of spraying ..... Date 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 thesediseases?.....
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 8

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 Sl. No.: .....
2. Name: .....
3. Age (years): .....
4. Gender: .....
5. Occupation: .....
6. Address: .....
7. Past history: .....

   a. Poisoning: Yes/No b. 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. Haemorrhagic 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/ Tobacco chewing/ 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 (00 /00 /0000)
Pulse rate/minute
Respiratory rate/minute
Depth of respiration
Temperature °F
(b) General
Pre-exposure
Weakness
Fatigue
Sleep

(Contd.....)

Urination					
Sweating					
(c) Gastro-intestinal					
	Pre-exposure	1 h	24 h	48 h	72 h
Nausea					
Vomiting					
Appetite					
Taste					
Abdomen pain					
Diarrhoea					
Sialorrhea					
(d) Neuro-muscular					
	Pre-exposure	1 h	24 h	48 h	72 h
Headache					
Dizziness					
Irritability					
Pain					
Twitching					
Tremors					
Convulsions					
Paraesthesia					
Hallucinations					
Unconsciousness					
(e) Cardio-respiratory					
	Pre-exposure	1 h	24 h	48 h	72 h
Nasal discharge					
Wheeze					
Cough					
Expectoration					
Chest tightness					
Dyspnoea					
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 /restlessness					
Insomnia					

X = No; N = Normal; NAD = Nothing abnormality 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 among spray-men

1. Time of recording and sample size—Study should be on at least 5 spray-men 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- Orthodromic 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.

Distancy.....cm ..... cm ..... cm

Conduction velocity .....metres/sec. (Wrist to elbow)

Conduction velocity .....metres/sec. (Elbow to supraclavicular region)

2. Right/Left ULL INar (Motor): Hypothenar muscles: Surf..... Ele.....

Wrist..... Elbow..... Supraclavicular.....Amp.....

Latency..... msec.....msec..... msec.....m.v.

Distancy.....cm ..... 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.

Distance .....cm ..... cm .....cm

Conduction velocity ..... metres/sec.

4. Right/Left Sural nerve (Antidromic-Sensory):NeelleEle.

Amplitude .....uv

Latency ..... msec

Distance .....cm

Conduction velocity ..... m/sec

5. Right/Left Median (Orthodromic Sensory)

Stimulation- digital nerves-index finger

Recording at wrist: Needle Ele./Surf. Ele.

Amplitude .....uv.

Latency .....msec

6. Right/Left Ulnar (Orthodromic 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..... Fasciculations .....
Insertional activity .....Myotonic .....

Interference pattern .....Amplitude of motor units .....

ii. Fibrillations..... Fasciculations .....

Insertional activity .....Myotonic .....

Interference pattern .....Amplitude of motor units .....

iii. Fibrillations..... Fasciculations .....

Insertional activity .....Myotonic .....

Interference 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:

* Only healthy volunteers/spraymen should be engaged.

Annexure 9

Consent form for human volunteers participating in the LLIN evaluation studies

ProjectTitle: .....

Name of the Institute and Address: .....

.....

Names of the responsible Investigators: .....

House No.: .....Village: .....

PHC/CHC: .....District: .....State: .....

I understand that 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. I understand that I will be required to act as bait for the studies to assess the impact of the IRS/ITN/LLIN/repellents. I also understand that I will be engaged as bait for the mosquitoes . The study will be conducted during night usually from dusk to dawn.

I am informed that the agent being used in the trial will not cause risk to human beings at the recommended dose.

I also understand that the Principal Investigator of the study can exclude me from the study if I do not adhere to the study procedures as described in the information sheet. I have been provided with a copy of this consent form.

However, I am also free to withdraw from the study without assigning any reason and without any implications thereof.

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction.

I am explained about the precautions that will be taken during the experiment and also assured of against any liability or risk and I agree to participate voluntarily.

If I have any further questions about the study, I should contact (Name of the Principal Investigator) or (Name of the Investigator) for reporting any discomfort or for immediate medical help (if needed).

Signature/thumb impression of the volunteer Date:

Signature of Principal Investigator/Investigator

(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 10

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 andwhy?:.....
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

    If Yes, provide reasons.....

    If No, provide 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 11

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

Title of the project:.....

Name of Principal 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 thisnet:.....
2. Caretaker of those using the net:.....
3. Head of household:.....
4. Other (specify) .....

Information on net usage:

1. Year-round and everynight
2. Year-round but occasionally
3. Seasonally but everynight
4. Seasonally and occasionally

How is the net used?

1. Hanging over thebed
2. Hanging over sleeping mat/mattress on theground
3. Other (specify).....

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

1) Yes 2) No

If Yes, describe the effect. .....

If No, 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. cold .... 2. 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. 1 Inside 2 Outside under shade 3. Outside under the sun

How was the net dried?

2. 1. Vertical 2. Horizontal

2. Physical inspection of nets

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. abitdirty
3. dirty
4. verydirty

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 or others.

   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 tear#

Name of investigator .....

Signature .....

#_____

This 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 misdemeanor (e.g. selling). If the remaining nets are quite holed, loss due to wear and tear would appear genuine.

Annexure 12

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 exposureetc.): .....
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-exposure and post-exposure: .....
13. Human toxicology proforma for liver and kidney function tests: .....

Liver function tests

1. Serum bilirubin: .....
2. SGOT: .....
3. SGPT: .....
4. Serum alkaline phosphatase: .....
5. Serum protein: .....

Kidney function tests

1. Blood urea: .....
2. Serum creatinine: .....
3. Other tests (if needed).....

Signature of Medical Officer/Physician

Date: (Seal)

Place:

10

Appendix

Appendix 1

Calculation of Doses

1. Measurement of sprayable surface area of a room

Formula = (L x W + W x H + H x L) x 2 – W x L

Example:

L: Length of wall = 12 ft

W: Width of wall = 10 ft

H: Height = 8 ft

Area = {(12 x 10 + 10 x 8 + 8 x 12) x 2} – (10 x 12)

= {(l20 + 80 + 96)x2} – 120

= (296 x 2) – 120

= 592 – 120

= 472 ft^2 (43.85 m^2)

Note: For measuring artificial surfaces and substrates 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 power (WDP) required for the preparation of approximately 10 litres of spray suspension.

The general formula followed

X = A x B x D / C

X = amount of water-dispersible powder required

A = percentage concentration desired

B = quantity of spray desired

C = percentage concentration of water-dispersible power

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

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

X = amount of dust required, A = dosage (kg/ha), B = percentage concentration of dust

Appendix 2

MODEL CALCULATION FOR FIELD APPLICATION (Spray spray)

Outdoor (Vehicle mounted equipment) :

Given Track spacing- 50 m;

Speed of vehicle - 12 Km/hr;

Application rate -500 ml/hectare

Linear distance = 50 m x 12000m/hr = 600,000 sq.m/hr or 60000/60 = 10,000sq m or 1 hectare/minute

If the application rate is 500 ml/hectare, the flow rate is to be adjusted @ 500ml/minute

If the flow rate is to be adjusted eg. 100 ml. minute, the speed of the vehicle is to be 12/5 (2.4 km)/hr for the track of 50 m.

Thus, the flow rate is determinant on product of (track X speed = area in sq,m expressed in hectares) and application rate using the following formula-

Flow rate (ml) = track (m) X speed (m/hr) x application rate (ml/min) ÷ 10,000 X 60

Outdoor (Portable equipment):

Given Track spacing- 10 m

Walking speed – 60 m/min

Application rate 500 ml/hectare

Linear distance = 10 m x 60 m/min = 600sq, m/min or 600/10,000 = 0.06 hectare/minute

If the application rate is 500 ml/hectare, the flow rate is to be adjusted to 30 ml/minute (500 ml x 0.06 hectare or 600 sq.m in 1 minute)

If the flow rate is to be adjusted eg. 15 ml. minute, the walking speed will be 120 m/min for the track of 10 m. to cover 600 sq m area of spray

Thus, the flow rate is determinant on product of (track X speed = area in sq,m expressed in hectares) and application rate.

Flow rate (ml) = track (m) X speed (m/hr) x application rate (ml/min) ÷ 10,000 X 60

Indoor application (dosage/room or house):

Given 400 sq.m (0.04 hectare) application area

Flow rate 20 ml/min

Application rate 500 ml/hectare

The equipment with adjusted flow rate of 20 ml/min will deliver 20 ml to cover 0.04 hectare (400 sq.m) in one minute.

If the flow rate is adjusted to 10 ml/min the application time for 400 sq.m will be 2 mi

Thus, flow rate = area in hectare x application rate

Note - Users should follow the label of the equipment for discharge rate and volume for coverage

Appendix 3

Measurement of surface area of mosquito breeding waters

(a) Rectangular/square area

Formula = L x W = Surface area

Example = 4.5 m x 3m = 13.5 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 x r x r

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

Radius of well/pit = 1.5 m

Area = 22/7 x 1.5 m x 1.5 m

= 3.1 x 1.5 x 1.5 = 6.97 m^2

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

Formula = πr^2x depth

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

Radius of well/pit = 1.5 m

Depth of well/pit = 0.30 m

πr^2x depth = 3.1 x 150 cm. x 150 cm x 30 cm = 2092500 cm

Volume of water = 2092500/1000 = 2092 litres of water

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

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

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

Width of breeding water = 25 m

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

$$\text{Or Area (acres)} = \frac{\text{L (ft) x W (ft)}}{43560}$$

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

Width of breeding water = 500 ft

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

Appendix 4

Measurements and Conversions

Volume

1 Liter = 1000 ml

1 ml = 1000 μl

1cubic meter = 1000 Liter

1 cubic foot = 7.5 gallons = 28 Liter

1 gallon = 4 quarts = 8 pints = 128 ounces = 3785 ml

Surface

1 ha = 10 000 m^2 = 2.2 acres

1 acre = 43 560 square feet

1 square foot = 0.111 square yard = 0.105 m^2

Length

1 km = 0.62 miles = 1093 yards
1 m = 100cm =39.7 inches
1 inch = 2.54 cm = 0.0254 m
1 foot = 0.3048 m =0.333 yards
1 yard = 91.44 cm = 0.9144 m
1 mile (statute) = 1760 yards = 5280 ft = 1609.3 m

Weight

1 pound = 0.454 kg

1 kg = 1000g =2.2 pounds

1 g = 1000mg=0.035 ounces

1mg= 1000μg

1 μg= 1000ng

Conversion factors

Square inches to square centimetres, multiply by 6.5.Square yards to square metres, multiply by 0.8.

Square feet to square metres, multiply by 0.09.Acres to hectares, multiply by 0.4.

Square miles to square kilometres, multiply by 2.6.

One microgram to nanogram, multiply by 100

Experts

Late Dr. Shiv Lal
Former Spl. DGHS, Former Director, National Center for Vector Borne Diseases Control &
Former Director, National Centre for Disease Control
New Delhi

Dr. P.L. Joshi
Former Director
National Center for Vector Borne Diseases Control
New Delhi

Dr. P. Jambulingam
Former Director
ICMR-Vector Control Research Centre
Puducherry

Drafting Committee

Dr. K. Gunasekaran
Scientist G (Retd.)
ICMR-Vector Control Research Centre
Puducherry

Dr. K. Raghavendra
Scientist G (Retd.)
ICMR-National Institute of Malaria Research
New Delhi

Dr. C. Sadanandane
Scientist D (Retd.)
ICMR-Vector Control Research Centre
Puducherry

Dr. Kalpana Baruah
Former Additional Director & Scientist 'V'
National Center for Vector Borne Diseases Control
New Delhi

Late Dr. Ram Singh
Joint Director
National Centre for Disease Control
New Delhi

Dr. Tanzin Dikid
Joint Director
National Centre for Disease Control
New Delhi

Dr. T. Sonai Rajan
Assistant Director (Entomology)
Central Insecticide Board and Registration Committee
Faridabad

Mr. Subhash Chand
Joint Director (Chemistry)
Central Insecticide Board and Registration Committee
Faridabad

Dr. Manju Rahi (Member Secretary)
Scientist G and Director
ICMR-Vector Control Research Centre
Puducherry
Programme Officer (VBDs)
Division of Epidemiology and Communicable Diseases
Indian Council of Medical Research
New Delhi

Technical Support

Dr. Sarala K. Subbarao
Former Director
ICMR-National Institute of Malaria Research
New Delhi

Dr. Ashwani Kumar
Former Director
ICMR-Vector Control Research Centre
Puducherry

Dr. A. N. Shriram
Scientist D
ICMR-Vector Control Research Centre
Puducherry

Dr. Vaishali Verma
Scientist C
ICMR-National Institute of Malaria Research
New Delhi

Dr. Gaurav Kumar
Technical Officer B
ICMR-National Institute of Malaria Research
New Delhi

Dr. Poonam Sharma Velamuri
Consultant (Non-Medical)
Indian Council of Medical Research
NewDelhi

Ms. Gurpreet Kaur Basra
Research Assistant
National Center for Vector Borne Diseases Control
New Delhi

Mr. Sandeep Rohilla
Sr. Technician
National Center for Vector Borne Diseases Control
New Delhi

Support

Dr. Sam Joy
Scientist C
Division of Epidemiology and Communicable Diseases
Indian Council of Medical Research
New Delhi

Dr. Jasmita Gill
Consultant, Publication Division
ICMR-National Institute of Malaria Research
New Delhi

Ms. Divya K
Former Scientist B
Indian Council of Medical Research
New Delhi

Notes

Notes

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Indian Council of Medical Research
2023