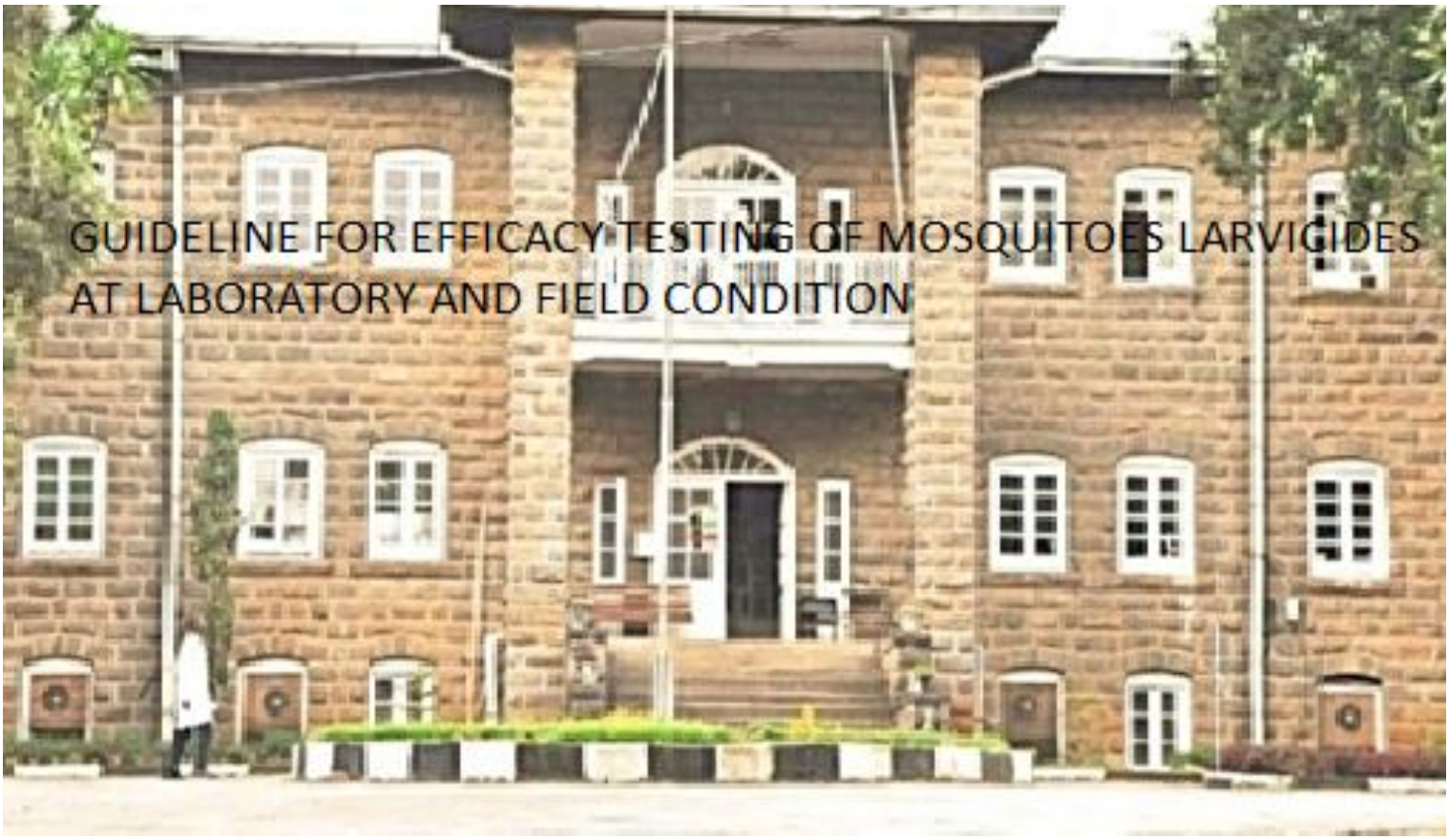

GUIDELINE FOR EFFICACY TESTING OF MOSQUITOES LARVICIDES AT LABORATORY AND FIELD CONDITION

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JUNE 2017

ADDIS ABABA, ETHIOPIA

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ABBREVIATIONS

ANOVA	Analysis of Variance
CDC	Center of Disease Control and Prevention
CI	Confidence Interval
EPHI	Ethiopian Public Health Institute
FMoH	Federal Ministry of Health
IE	Inhibitor Emergence
IRS	Indoor Residual Spray
ITU	International Toxic Units
IVM	Integrated Vector Management
LC	Lethal Concentration
LLIN	Long Lasting Insecticide Net
MoANR	Ministry of Agriculture and Natural Resource
PHPs	Public Health Products
VBD	Vector Borne Disease
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme

1. INTRODUCTION

Vector borne diseases (VBD) are emerging or resurging as a result of changes in public health policy, insecticide and drug resistance, demographic and societal changes, and genetic change in pathogens**.** In 2015 malaria cases and deaths declined with 18% and 48% respectively. Most of the cases were occurred in WHO African region (WHO, 2015). The wide-scale deployment of malaria control interventions brings 108malaria endemic countries in 2000 to 91 countries in 2016 (WHO, 2016). Malaria is also one of the main public health problems in Ethiopia where 75% of the geographic area has significant malaria transmission risk (Aschalew*et.al*, 2016).

Mosquitoes are one of the most important public health pests, capable of transmitting diseases such as malaria, yellow fever, dengue fever, filariasis and Japanese encephalitis and some other viral diseases to humans and animals (Mukhtar, 2010). Malaria parasite is transmitted with the bite of female mosquitoes of the genus *Anopheles* (Ayele, 2016). *Anopheles gambiae* complex is the main malaria vector in Sub-sahara Africa (Onyabe, 2001; Moralis, 2005). In Ethiopia, there are about 45 *Anopheles* mosquito species of which *Anopheles gambiaes.l* is the most prevalent (Ayele, 2016). *Anopheles arabiensis* is the main malaria vector in Ethiopia and *An. funestus, An. pharonsis* and *An. nilli* are considered as secondary vectors (Kenea, *et.al*, Taye *et.al*, 2016*).* Mosquitoes are often difficult to control because their wide range of breeding habitats, resting places and resistance issue. Use of insecticides alone or any single method will not control mosquitoes significantly, (Mukhtar, 2010).

Vector control is an important component to interrupt malaria transmission. Malaria vector control has more than 50 year‟s history in Ethiopia (Aschalew, 2016). Currently, Ethiopia uses LLINs and IRS as the main vector control tools. However, the development and spread of malaria vector resistance to pyrathroids and IRS chemicals are becoming challenges to the current vector control interventions and contradicts malaria elimination strategic plan. To prevent mosquito-borne diseases and improve public health, it is necessary to control them (Abdul etal, 2010). As the use of insecticides alone or any single method will not control mosquitoes significantly, (Mukhtar, 2010), IVM approach is vital and universally accepted to achieve the national and global targets set for vector-borne disease control (WHO, 2012*).* IVM is the use of a range of control interventions such as LLINs, IRS, environmental management and larviciding in combination or alone. Effective larvicides can reduce the number of adult mosquitoes available to disperse, potentially spread disease; create a nuisance, and lay eggs which leads to more mosquitoes. Unlike IRS and LLINs which targets only indoor adult mosquitoes, larviciding has a dual benefits in which it reduce both the number of indoor and

outdoor mosquitoes (IRAC, 2014). Before application of larviciding it is critical to have a thorough knowledge of the biology of the targeted species in order to determine the appropriate larvicide, the timing of the application, and the amount of product to be applied. The efficacy of larvicide should also be evaluated under laboratory and field conditions. To evaluate efficacy of larvicide and generate valuable data, there should be standardized protocol. Thus, the purpose of this document is to provide specific and standardized operational procedures (SOP) and guidelines for testing natural products based and synthetic chemicals larvicides, including bacterial larvicides against malaria vectors in Ethiopia.

2. SCOPE AND OBJECTIVES

Larvicides are used for vector control under disease control programs. Larvicides include chemical larvicides, natural product based larvicides bio-larvicides and insect growth regulators. Evaluation of larvicides is carried out for both WHOPES passed and new insecticides. New insecticides are evaluated in three Phases - I, II and III, while for WHOPES passed insecticides only Phase II and III trials are conducted to conform evaluation requirements. Information collected through entomological surveillance techniques assist in the understanding of the spatial and temporal changes in vector species, efficacy and effectiveness of vector control measures employed for malaria vector control. EPHI is responsible to evaluate efficacy of vector control intervention tools at different sentinel sites under Ethiopia condition and generates data for FMoH and MoANR. Based on EPHI data Ministry of agriculture and Natural resource has the mandate of registering and allows introducing new PHPs (which include insecticides/ insecticide formulations/ LLINs/ bio-larvicides) for vector control under national program on the basis of their suitability and adaptability.

For the sake of meaningful comparison of results generated at different sentinel sites in terms of deciding the suitability of the products for Ethiopian conditions, development of WHO based standard institutional guidelines for larvicide efficacy testing has become imperative. The trials conducted by the institution at different sentinel sites following such guidelines/protocol will minimize the discrepancies in the methodology and thereby their results could be compared more meaningfully to facilitate the FMoANR to arrive at decision. This protocol follows WHO guidelines and it includes:-

Phase I, Laboratory studies: - Bio potency and activity, Diagnostic concentration and assessment of cross-resistance,

Phase II, Small scale field trials: - Efficacy under different ecological settings, Method and rate of application, Initial and residual activity and Effect on non-target organisms and

Phase III, Large-scale field trials: - Efficacy and residual activity, Operational and community acceptance, effect on non-target organisms to determine the efficacy, field application rates and operational feasibility and acceptability of a mosquito larvicide. The guide line does not address evaluation of all biological control agents other than bacterial Larvicide.

3. CHEMICAL LARVICIDES

3.1. PHASE I: LABORATORY STUDIES

Laboratory efficacy of new technical PHPs or their formulations is done under controlled conditions using laboratory-reared vector species. This phase includes studies on efficacy and persistence, diagnostic dosage and cross-resistance in vectors. To determine the inherent Bio potency of the technical material or, in the case of formulated mosquito larvicides and their activity, laboratory testing is the first mandatory procedures. Information on the speed of activity is important, as this will determine the type of testing procedures to be employed.

Laboratory-reared mosquito larvae of known age or instar are exposed for 24 h in water treated with the larvicide at various concentrations within its activity range, and mortality is recorded, to confirm the biological activity of a mosquito larvicide. For IGRs and other materials with delayed activity, mortality should be assessed until the emergence of adults. It is important to use homogenous populations of mosquito larvae or a given instar.

3.1.1. Objectives of the test:

- To establish dose–response line(s) against susceptible vector species;
- To determine the lethal concentration (LC) of the larvicide for 50% and 90% mortality (LC50 and LC90) or for 50% and 90% inhibition of adult emergence (IE50 and IE90);
- To establish a diagnostic concentration for monitoring susceptibility to the mosquito larvicide in the field; and
- To assess cross-resistance with commonly used insecticides.

3.1.2. Determination of biological activity

In order to detect the biological activity of a mosquito, it is necessary to establish a base-line for the species of a given insecticide with samples from laboratory reared mosquitoes.

4. MATERIALS AND REAGENTS

4.1. Materials

- ✓ One (1) pipette delivering 100-1000µl
- ✓ Disposable tubes (100µl, 500 µl) for measuring aliquots of dilute solutions
- ✓ Five (5) 1ml pipettes for insecticides and one for the ethanol
- ✓ Three droppers with rubber suction bulbs
- ✓ Strainer: Strainer• made from two wire loops, one piece of nylon netting (30 cm^2) and one tube of cement. It is suggested that two pieces of netting be cut and cemented to opposite sides of the larger end of the wire loops. More cement should then be applied around the edges of the loops to join the two pieces of netting. When dry, the netting may be trimmed with scissors. If a strainer is not available, a loop of plastic screen may be used to transfer test larvae into test cups or vessels.
- ✓ Disposable cups or, glass bowls/beakers capacity of 100ml and 250ml
- ✓ Measuring cylinder
- ✓ Log-probit papers
- ✓ Data record forms
- ✓ Top-drive homogenizer or stirrer for lyophilized products
- ✓ Ice bath (container of crushed ice) for grinding or sonication
- ✓ Micropipette
- ✓ 10 ml pipette
- ✓ 12 ml plastic tubes with stoppers or caps
- ✓ 120 ml or 250 ml plastic or wax-coated paper cups to hold
- ✓ 100 ml or 200 ml water

4.2. Reagents

- ✓ Insecticide(s) to be tested (technical grade or formulations);
- ✓ Acetone or technical grade absolute ethanol

4.3. Biological material

- ✓ Mosquitoes Larvae for testing

5. PREPARATION OF TEST CONCENTRATIONS AND STOCK SOLUTIONS OR SUSPENSIONS

Prepare the test concentration (stock solution) by pipetting 1ml of the standard insecticide solution under the surface of the water in each of the beakers and stirring vigorously for 30 sec. with the pipette. The technical materials of many organic compounds are insoluble in water. These materials have to be dissolved in appropriate organic solvents such as acetone or ethanol in order to prepare dilutes of series concentration of working solutions for laboratory testing. The formulated materials are, however, miscible with water. Suspending or mixing these formulations in water requires no special equipment homogeneous suspensions can be obtained by gentle shaking or stirring. In preparing a series of concentrations, the most dilute should be prepared first. Four or more replicates are set up for each concentration and equal numbers of controls are set up simultaneously with water in which 1ml ethanol is added. Test concentrations are then obtained by adding 0.1–1.0 ml (100–1000 µl) of the appropriate dilution to 100 ml or 200 ml distilled water (WHO, 2005.13).

5.1. Stock solution Volume

- ✓ It should be 20 ml of 1% gained from 200 mg of the technical material in 20 ml solvent (Tap water).
- ✓ It should be kept in a screw-cap vial, with aluminium foil over the mouth of the vial.
- ✓ Shake vigorously to dissolve or disperse the material in the solvent.
- ✓ The stock solution is then serially diluted (ten-fold) in ethanol or Acetone (2 ml solution to 18 ml solvent).

Small volumes of dilutions should be transferred to test cups by means of pipettes with disposable tips. The addition of small volumes of solution to 100 ml, 200 ml or greater volumes of water will not cause noticeable variability in the final concentration. When a test is carried out

using formulated materials, distilled water is used in the preparation of the 1% stock solution or suspension and in subsequent serial dilutions, according to the content of the active ingredient.

6. PROCEDURES FOR BIOASSAYS

- Expose the mosquito larvae to a wide range of test concentrations and a control or to find out the activity range of the materials under test.
- After determining the mortality of larvae in this wide range of concentrations, a narrower range (of 4–5 concentrations, yielding between 10% and 95% mortality in 24 h or 48 h) is used to determine LC50 and LC90 values.
- Transfer batches of 25 third or fourth instar larvae by means of strainers, screen loops or droppers to small disposable test cups or vessels, each containing 100–200 ml of water.
- Small, unhealthy or damaged larvae should be removed and replaced.
- The depth of the water in the cups or vessels should remain between 5 cm and 10 cm; deeper levels may cause undue mortality.
- The appropriate volume of dilution is added to100 ml or 200 ml water in the cups to obtain the desired target dosage, starting with the lowest concentration.

Four or more replicates are set up for each concentration and an equal number of controls (negative controls) are set up simultaneously with the vehicle used to prepare the testing sample i.e., tap water, to which 1 ml alcohol (or the organic solvent used) is added.

- Each test should be run three times on different days.
- For long exposures, larval food should be added to each test cup, particularly if high mortality is noted in control.
- The test containers are held at 25–28 ^{o}C and preferably a photoperiod of 12 h light followed by 12 h dark (12L:12D).
- After 24 h exposure, larval mortality is recorded.
- For slow-acting insecticides, 48 h reading may be required.
- Moribund larvae are those incapable of rising to the surface or not showing the characteristic of diving reaction when the water is disturbed and counted and added to dead larvae for calculating percentage mortality. Dead larvae are those that cannot be

induced to move. The results are recorded on the form provided, where the LC_{50}, LC_{90} and LC_{99} values, and slope and heterogeneity analysis are also noted.

- The form will accommodate three separate tests of six concentrations, each of four replicates.
- Larvae that have pupated during the test period will negate the test.
- If more than 10% of the control larvae pupate in the course of the experiment, the test should be discarded and repeated.
- If the control mortality is between 5% and 20%, the mortalities of treated groups should be corrected according to Abbott's formula given below:

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

Where X = percentage survival in the untreated control

Y = percentage survival in the treated sample

The probit mortality per log dose regressions for two insecticides could be compared using a parallelism test, (WHO, 2005.13/ICMR/NVBDCP, 2010).

6.1. Data analysis

- Data from all replicates should be pooled for analysis.
- LC_{50} and LC_{90} values are calculated from a log dosage probit mortality regression line using computer software programs, or estimated using log–probit paper.
- LD_{50} and LD_{90} and their 95% confidence limits are determined using commercial software
- Bioassays should be repeated at least three times, using new solutions or suspensions and different batches of larvae each time.
- Standard deviation or confidence intervals of the means of LC_{50} values are calculated and recorded (A test series is valid if the relative standard deviation (or coefficient of

variation) is less than 25% or if confidence limits of LC_{50} overlap (significant level at $P < 0.05$).

- The potency of the chemical against the larvae of a particular vector and strain can then be compared with the LC_{50} or LC_{90} values of other insecticides.

6.2. Determination of diagnostic concentration

Diagnostic dose is determined by multiplying the upper fiducially limit of $LC_{99.9}$ with a factor of 2 for routine susceptibility test (The diagnostic concentration is double that of the estimated $LC_{99.9}$ value).

6.3. Cross-resistance assessment

To assess the cross-resistance to other insecticides currently in use in the program, bioassays should be done using the diagnostic dosage of the test larvicide and of other larvicides. Susceptible strains of some mosquito species are kept in laboratories. Otherwise, any susceptible strains should be collected in the field (if truly susceptible populations still exist). If not, susceptible strains may be artificially selected using bioassays, assays for individual resistance mechanisms and selection between lines derived from individually mated females. The resistant strains should be identified using well established assay techniques. The strains should preferably be homozygous for one or more known resistance mechanisms. If homozygosity cannot be achieved, periodic selection is usually necessary to prevent natural selection favoring the susceptible at the expense of the resistant (WHO, 2005.13).

6.4. HASE II: SMALL-SCALE FIELD TRIAL (MULTI-CENTRIC

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

6.4.1. Objectives

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

6.4.2. Trial in natural breeding habitats

In natural breeding habitats of the target species, the field efficacy of the larvicide is tested. Selection of habitats for the testing is done based on all the major types of habitats and habitats selected should be similar and comparable in terms of vectors prevalence and their density. For Anopheles species, cement tanks, drums, garden pits, pools, rice plots, river/ stream bed pools, water fountains and disused wells; for Culex species stagnant drains, cesspits, cesspools and disused wells and for Aedes spp. cement tanks, drums, peri-domestic water storage containers and water fountains are best suited. At least three replicates of each type of habitat should be randomly selected for each dosage of the larvicide/formulation, with an equal number of controls. Depending on the surface area and depth, the size of the habitat is recorded. Each of the confined breeding sources or containers can be considered as a discrete habitat or replicate. Habitats such as canals may be divided into sectors of 10 m length and replicated for treatment and control. Prior to application of larvicidal formulation, density of larvae and pupae should be monitored for a week at least on two occasions. Breeding habitats from each type with comparable pre- treatment densities should be allotted equally to treatment and control groups. Density of larvae and pupae in the selected habitats is recorded prior to treatment by taking samples using a standard dipper (9 cm diameter with 300 ml capacity) (dipper method) for pits, ponds, tanks, drains, drums etc., or a bucket (3 liter capacity) for wells.

Number of samples from each habitat is determined based on the size of the habitat. sampling at a distance of 3 to 5 dipper samples are recommended for small habitats such as drums, pits, pools, tanks and wells, per habitat, while 3-5 m are recommended for stagnant drains. The larval instars and pupae collected from each dipper sample are counted and recorded stage-wise and returned to the habitats. Observation is made on the presence of non-target organisms

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

To determine the application rate:

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

To calculate the required concentration of larvicide suspension:

$$\text{Concentration of larvicide (Emulsion/suspension)} = \frac{\text{Dosage to be applied (g/m)}2}{\text{(Application rate (ml/m) 2}} x100$$

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

6.4.3. 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 habitats using Mulla‘s formula. The difference between treatments can be compared by two way analysis of variance (ANOVA) with treatment and number of days as independent factors. The ANOVA should be carried out after transforming the percentage reduction to arcsine values. The post-treatment day up to which 80% or 90%

reduction is observed at 95% CI for each treatment or dosage will then be compared to determine the residual effect and optimum application dosage for the Phase III trial.

$$\% \text{ Reduction } = 100 - \frac{\text{C1 x T2}}{\text{T1 x C2}} x\ 100, \text{ Where,}$$

C1 = Pre-treatment immature density in control habitats

C2 = Post-treatment immature density in control habitats

T1 = Pre-treatment immature density in treated habitats

T2 = Post-treatment immature density in treated habitats

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.

6.4.4. Trial in simulated field condition

Testing is done in simulated conditions against the mosquito species that breed in domestic and peri-domestic clean water habitats and can be carried out in containers (drums, jars, buckets, tubs, etc.). Against *Cx. quinquefasciatus*, testing is not carried out in simulated field condition. The efficacy of the larvicidal formulations is tested against laboratory reared *An. Arabiensis and Ae. Aegypti Linnaeus (Diptera Culicidae)* larvae under simulated field conditions. Cement tubs with 100 lit or 200 lit capacities (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 one up to two week. The tubs are then emptied, scrubbed, rinsed thoroughly with water and dried for a day – two weeks. The tubs are placed under a shed having only a roof and open on all sides, simulating the field condition. The placement of the tubs is configured in a block design form to equally distribute positional effects. The tubs are filled with domestic tap water (100 lit or 200

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

Two regimens of water can be used: In the first regimen, tubs are kept full for the duration of the experiment without removing the water; and in the second regimen, half of the water in the tubs is removed and replenished weekly with fresh tap water to simulate water use conditions. The two regimens of water are used for control as well as treatment. To assess the efficacy, a batch of 25-100 laboratory-reared early fourth instar larvae are released into each cement tub or replicate. To each tub, 0.5 gm ground up larval food is provided initially before adding first cohort of larvae and weekly thereafter. After 2-3 h of larval acclimatization, the tubs are treated with the larvicide/ formulation at 3-5 dosages in a randomized manner. In each water regimen, a minimum of four replicates of each dosage and four controls should be used. The water level in the tubs must be sustained. All the containers are examined after 48 h of treatment and live larvae are counted to score post-treatment larval mortality. To test residual activity, the treatments are challenged with new cohorts of larvae (early third instar) of the same mosquito species weekly and larval food is provided on alternate days or weekly. Larval and pupal survival is assessed 48 h post- treatment and data are recorded on. 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.

6.4.5. 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 >85% reduction in the pre-treatment counts (% reduction is calculated using the Mulla‘s formula, stated section 6.4.3). 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 85% mortality (and its 95% CI) (the desired level of control) is achieved for a given dosage. The effective duration of the dosages tested under the Phase II trial in natural or simulated habitats will be compared and the lowest dosage that produces the maximum effective duration will be selected as the optimum field application dosage for the Phase III trial.

6.5. PHASE III: LARGE-SCALE FIELD TRIAL (MULTI-CENTRIC)

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

6.5.1. Objectives of the trial are:

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

6.5.2. Selection of study area

Depending on type of larval habitat and the environment, locality of one square kilometer of urban/ rural area is selected for treatment and a locality of similar type and size for control. All types of breeding habitats are surveyed to ascertain the breeding of the target species in order to decide the suitability of the locality for the trail. A selected locality should have a minimum of 25–30 replicates or plots of each type of larval habitat of the target species should be selected for control and then again for treatment larval habitat positivity for the target mosquito species. Care should be taken. As in the case of small-scale field trials, each of the confined breeding habitats can be considered as a discrete habitat or replicate. Habitats such as drains and canals may be divided into sectors of 10 m^2 length and replicated for treatment and control.

6.5.3. Assessment of pretreatment of density

Prior to treatment, density of larvae and pupae is assessed in treatment and control habitats on at least two occasions during a week. The immature density is measured in different types of habitats using appropriate sampling devices (as given in Section 6.4.2).

6.5.4. Application of larvicidal formulation

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

6.5.5. Assessment of density after treatment

By taking fixed number of samples from the treated and control habitats at 24 and 48 h post-treatment and thereafter at weekly intervals, the density of larvae and pupae is assessed. Sampling procedures are similar to those followed for small-scale field trials in natural breeding habitats. Observations will be made for a minimum of three treatments for each trial and the trials should be repeated at least three times in different seasons. Data should be recorded on the Annexed prescribed form.

6.5.6. Impact on adult density and non-target organisms

By measuring the density of adult mosquitoes resting indoors (human dwellings/ animal sheds) using hand catch method. At least 7-10 dwellings selected randomly will be used for estimating the adult density per man-hour in the treated and control areas to provide information on the trend in the reduction of adult population of target mosquito species due to the effect of the larvicidal application. For assessing the impact of the larvicidal application on non-target organisms that co-habitat with mosquito immature, their density can be monitored while

sampling mosquito larvae and pupae during the large scale field trial. Larvivorous fish, snails, mayfly naiads, dragonfly naiads, copepods and aquatic beetles are some of the common non-target organisms that co- habitat with mosquito larvae. If non-target effect will be studied and also stated under the objective, the methods should be clearly stated.

6.5.7. 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

6.5.8. 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 6.4.3. Will be followed to confirm the residual efficacy and frequency of application.

7. BACTERIAL LARVICIDES

7.1. PHASE I: LABORATORY STUDIES

7.1.1. Backgrounds

Larval control appears promising in urban areas generally, given the high density of humans needing protection and the limited number of breeding sites. For example, the vector *An. gambiae* is capable of breeding in small puddles of rainwater and may not be controllable in rural areas, where the number of potential breeding sites is enormous. However, under certain circumstances, populations of this species may concentrate in a few sites (e.g., construction borrow pits) in urban areas. Agents that effectively suppress larval populations under laboratory

conditions often fail under less favorable field conditions (Kathleen W., 2002). As mosquito species become resistant to the chemical larvicides currently available, and people become increasingly concerned about the impact of chemicals on the environment, interest in the use of bacterial larvicides as alternative control agents for mosquitoes has increased.

The most widely used entomopathogens in mosquito control are commercially produced bacteria. Larvicides containing these bacteria are harmless to beneficial insects and animals, but can rapidly kill susceptible larvae, which are the immature stages of the mosquito that live in water. Two different species of bacteria of the genus Bacillus, *B. thuringiensis israelensis* (Bti) and *B. sphaericus* (Bs), have been widely demonstrated to be effective larvicides against both *anopheline* and other mosquito species. Both Bti and Bs function as stomach poisons in the mosquito larva midgut. Since the discovery of the mosquito larvicidal activity of Bti spores (serotype H-14) in 1977, different formulations of Bti have been found effective against larvae of many mosquito species, including the malaria vectors *An. albimanus*, *An. sinensis*, *An. culcifacies*, *An. sundaicus*, *An. stephensi*, *An. gambiae*, *An. arabiensis*, and *An. Maculatus.*

7.1.2. Objectives of the trials

- To establish dose-response relationships of the given bacterial larvicide(s) against the target vector species,
- To determine LC_{50} and LC_{90} and dosages for Phase II trial

7.1.3. Bioassay Principles

The laboratory bioassay procedures for bacterial products are the same as those for chemical larvicides, but different in their preparation of stock suspensions. Duration of effectiveness mainly rely primarily on the mosquito species, the environmental conditions, the formulation of the product, and water quality. The bio potency of the material is first examined by comparing mosquito larval mortality produced by the product under test with the mortality produced by the corresponding reference standard or other technical or formulated product. The toxicity of preparations based on *Bacillus thuringiensis* subsp. *israelensis (B. thuringiensis* subsp.

israelensis) can be determined against a standard product that has been calibrated using *Aedes aegypti (A. aegypti)* larvae. The potency of products tested is determined by the following formula (WHO, 2005.13)

$$\text{Potency of product “X”} = \frac{\text{Potency standard (ITU) x LC50 (mg/l)standard}}{\text{LC50 (mg/l) of “X"}},$$

When the international reference standard is used, potency is expressed in International Toxic Units per milligram (ITU/mg). The bio-potency of products based on *B. thuringiensis* subsp. *israelensis* compared with a lyophilized reference powder (IPS82, strain1884) of this bacterial species using early fourth instar larvae of *A. aegypti* (strain Bora Bora). The potency of IPS82 has been arbitrarily designated as 15 000 ITU/mg powder against this strain of mosquito larva. The bio-potency of products based on *Bacillus sphaericus* (*B. sphaericus)* is determined against a lyophilized reference powder (SPH88, strain 2362) of this bacterial species using early fourth instar larvae of *Culex pipiens pipiens (C. pipiens pipiens)* or *Culex quinque fasciatus*. The potency of SPH88 has been arbitrarily set at1700 ITU/mg of powder against this mosquito strain.

The use of other bacterial larvicide reference powders and/or alternative strains of mosquito in this test is possible but must be approached warily, because it is inevitable that different results will obtain. Such alternatives must be the subject of careful cross calibration with the reference powders and strains identified above. Ideally, such cross-calibration should be conducted by a group of independent expert laboratories. The alternative powders or strains, and the cross-calibration data that support them, should be made available to anyone who wishes to use, or check, the test. In general, it is not necessary to calibrate with or test against the standard if comparing the activity of a bacterial product with other larvicide products. Bioassay results providing LC50 and LC90 values of products are sufficient to enable comparison among different products.

7.1.5. Preparation of reference standard suspensions for calibration of the bioassays

For conducting laboratory evaluation of the candidate biolarvicide, the first step will be to prepare a stock solution, normally 1%.

1. Weigh 200 of solid mg or 1000 mg of the solid product,
2. Place in a vial (30 ml) or volumetric flask,
3. Add 20 ml or 100 ml distilled water to it, yielding 1% stock suspension, or 10 mg/l.
4. Most powders do not need blending or sonication.
5. Shake or stir vigorous to facilitate suspension.
6. Place stock suspension in 17 tubes, to frozen it for future bioassays.
7. Thoroughly homogenize frozen aliquots before use, because particles agglomerate during freezing.
8. The stock solution is then serially diluted (ten-fold) in distilled water.
9. 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.

7.1.6. Laboratory bioassays:

The laboratory bioassay procedures for bacterial larvicides are the same as those for chemical larvicides (Please refer to Section 6). Mortality is scored at 24 hours post-treatment for *B. thuringiensis* subsp. israelensis and 48 hours for *B. sphaericus* by counting the live larvae remaining in the test cups. When the exposure period is 24 h, addition of larval food to the test cups is not required and if the exposure period is extended to 48 hour food may be added. The results are recorded on the form.

7.1.7. Data analysis

LC50 and LC90 of the candidate biolarvicide will be calculated from mortality regression lines by probit analysis. The dosages for Phase II trials are calculated based on LC99.9 values determined under Phase I trial.

7.2. PHASE II: SMALL-SCALE FIELD TRIAL (MULTI-CENTRIC)

Bacterial larvicides that show promising activity in the laboratory studies (Phase I) are subjected to small-scale field testing (Phase II). The application dosages for the Phase II trial will be determined by multiplying the observed LC99.9 value with a factor of 2 and above so as to obtain at least a range of 3-5 dosages for small scale testing.

7.2.1. 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.

7.2.2. Trial in natural breeding habitats

The small-scale dose determination trial procedures for bacterial larvicides are the same as those for chemical larvicides (Section 6.4.2). Trial in simulated field condition and Data analysis are the same as those for chemical Larvicides.

7.3. PHASE III: LARGE-SCALE FIELD TRIAL (MULTI-CENTRIC)

In phase III, 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.

7.3.1. Objectives

- To confirm the efficacy of the bacterial larvicide applied at the selected field application dosage(s) against the target mosquito species in natural breeding sites
- To confirm residual activity and application intervals

- To record observations on the ease of application and dispersal of the insecticide
- To observe community acceptance
- To record any perceived side-effects on operators and
- To observe the effect of the treatment on non-target organisms.

7.3.2. Evaluation

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

14.REFERENCES

1. Abdul Abduz Zahir, Abdul Abdul R. Asokan Bagavan, Gandhlango, Chinnaperumal Kamaraj, (2010). *Adult emergence inhibition and adulticidal activities of medicinal plant extracts against Anopheles stephensi Liston*. Asian Pacific Journal of Tropical Medicine. Journal homepage: www.elsevier.com/locate/apjtm. India.
2. Aschalw, A. and Tadesse, D., (2016). Current status of malaria in Ethiopia: Evaluation of the burden, factors for transmissions and prevention methods., 7(1),pp.1, pp.1-6
3. Ayele (2016). *Distribution of the main malaria vector in Ethiopia*: Malaria Journal 9:69, Anopheles species and malaria transmission risk in a highland area, south-central Ethiopia. Addis Ababa.
4. Centers for Disease Control and Prevention (CDC). *Guideline for Evaluating Insecticide Resistance in Vectors Using the CDC Bottle Bioassay*. http://www.cdc.gov/malaria. Atlanta, USA. Accessed on 20th April 2017.
5. Centers for Disease Control and Prevention, (2005). *Public Health Confronts the Mosquito, Developing Sustainable State and Local Mosquito Control Programs*. Washington, DC. www.ASTHO.org, www www.StatePublicHealth. Accessed on 20th April 2017
6. Department of Pesticide Regulation (DPR), (2017). *A Guide to Pesticide Regulation in California: California Environmental Protection Agency*. www.cdpr.ca.gov. California
7. Indian Council of Medical Research, National Vector Borne Disease Control Program and National Centre for Disease Control, (2014). *Revised Common Protocol for Uniform Evaluation of Public Health Pesticides including Bio-larvicides for use in Vector Control*. India, New Delhi.
8. Inecticide Resistance Action Committee (IRAC), (2014). *Theory and practice of mosquito larviciding.*
9. K. John McConnell1 and Duane J. Gubler, (2003). *Guidelines on the cost-effectiveness of larval control programs to reduce dengue transmission in Puerto Rico, Cost-effectiveness of larval control programs to reduce dengue transmission in Puerto Rico*. United States of America.
10. Kathleen Walker (2002). *A Review of Control Methods for African Malaria Vectors: Environmental Health Project*. Bureau for Global Health, U.S. Agency for International

Development, under EHP Project 26568/CESH.OPR.MAL.LI. U.S. Agency for International Development. Washington, DC.

11. Kenea O., Balkew M., Tekie H., Gebreminchael T., Derssa W., Loha E., Lindtjorn B. and Overgard J.H., (2016). *Human-biting activities of Anopheles species in south-central Ethiopia*. Parasites & Vectors.
12. Mir S., Mulla and Charles H. Schaefer (1980). *Developing better larvicides*: Department of Entomology. University of California, Fresno Mosquito Control Research Laboratory, CALIFONIA AGRICULTURE. CALIFORNIA
13. Moralis I. and Frederic S., (2005). *Population structure of Anopheles arabiensis onlareunion island, Indian Ocean*.
14. Mukhtar M., (2010). *Guidelines for control of vectors of public health importance after monsoon rains in Pakistan*. Department of Zoonotic and Vector-Borne Diseases Epidemic Investigation Cell, Public Health Laboratories Division National Institute of Health. Ministry of Health. Government of Pakistan.
15. Nirman Bhawan, (2016). *Operational Manual for Malaria Elimination in India*, Ministry of Health & Family Welfare. Government of India. New Delhi.
16. Onyabe, D.Y. and Conn, J.E., (2001). *The Distribution of Two Major Malaria Vectors, Anopheles gambiae and Anopheles arabiensis, in Nigeria*. *Memorias do Instituto Oswaldo Cruz*.
17. PMI, (2012). *National Policy Guidelines for Integrated Vector Management*: Integrated Vector Management (IVM). Ministry of Health. Rwanda.
18. Sham NathMarg, (2007). *India environmental management plan for the vector borne disease control project under World Bank*: Ministry of Health & Family Welfare, www.nvbdcp.gov.in. New Delhi.
19. Taye B., Lelisa K., Emana D., Asale A. and Yewhalaw D., (2016). *Seasonal Dynamics, Longevity, and Biting Activity of Anopheline Mosquitoes in Southwestern Ethiopia*. *Journal of Insect Science*. Addis Ababa.
20. The Environmental Protection Agency (EPA), (2000). *For your information Larvicides for Mosquito Control*. United States Environmental Protection Agency and Prevention, Pesticides and Toxic substance. Web site: http://www.epa.gov/pesticides or http://www.cdc.gov. Atlanta.

21. UN Medical Services Division, (2016). *Best Management Practice for Mosquito Control Program for UN Duty Stations*. http://www.who.int/whopes/Mosquito_Larvicides_25_Oct_2013.pdf. Accessed on 17th April 2017.
22. World Health Organization (WHO), (1996). WHO pesticide evaluation Scheme: *Report of the WHO informal consultation on the evaluation and testing of insecticides (WHOPES), Division of control of tropical diseases*. Geneva.
23. World Health Organization (WHO), (1999). *Guideline specifications for bacterial larvicides for Public Health Use*: Report of the WHO informal consultation. Geneva.
24. World Health Organization (WHO), (2006). *Pesticides and their application for the control of vectors and pests of public health importance, Sixth edition*. Department of Control of Neglected Tropical Diseases WHO Pesticide evaluation scheme (WHOPES. Geneva.
25. World Health Organization (WHO), (2012). *Handbook for integrated vector management*. (www.who.int). Geneva.
26. World Health Organization (WHO), (2013). An Operational Manual: *Larval Source Management, A supplementary measure for malaria vector control*. Geneva, Switzerland. (www.who.int/malaria). Accessed on 15th April 2017.
27. World Health Organization (WHO), (2015). *World Malaria Report*. Available at: http://www.who.int/malaria/publications/world-malaria-report-2015/report/en.
28. World Health Organization (WHO), (2016). *World malaria report. Available* at: http://apps.who.int/iris/bitstream/10665/252038/1/9789241511711-eng.pdf?ua=1.
29. World Health Organization Communicable Disease Control, Prevention and Eradication, (2003). *WHO Pesticide Evaluation Scheme (WHOPES, Draft guidelines on the management of public health pesticides*: Report of the WHO inters regional consultation chiangmai, Thailand.
30. World Health Organization Communicable Disease Control, Prevention and Eradication WHO pesticide evaluation scheme, (2005.13). *Guidelines for Laboratory and Field testing of Mosquito Larvicides*. Geneva.
31. World Health Organization, (2006). WHO Technical Report Series: *Malaria Vector Control and Personal Protection*. Report of a WHO Study Group, Geneva, Switzerland

ANNEX 1. **DILUTION AND CONCENTRATIONS**

Aliquots of various strength solutions added to 100 ml water to yield final concentration

Initial solution		Aliquot (ml)[a]	Final concentration (PPM) in 100 ml
%	PPM		
1.0	10 000.0	1.0	100.0
		0.5	50.0
		0.1	10.0
0.1	1 000.0	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.

ANNEX 2. LABORATORY EVALUATION OF THE EFFICACY OF LARVICIDES.

Experiment No:_____ Investigator: _____

Treatment date: _____ Larvicide: _____

Test species: _____ Larval instar: _____

Temperature: _____ Lighting: _____

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

LC50 (CL 95%): _____
LC90 (CL 95%): _____
LC 99.9: _____

Slope: _____ Heterogeneity: _____

ANNEX 3. FIELD EVALUATION OF LARVICIDES

Small-scale field testing and evaluation of larvicides against mosquito larvae

Experiment No: _____ Starting date: _____ Location: _____ Investigator: _____
Assessment date: _____ Pre or days posttreatment: _____ Types of Habitat:_____Species _____

		Live larvae (L3-4) and pupae (P)/sample					
Treatments		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Grand total
Dosage ( )	Sample	L3-4* P*	L3-4 P	L3-4 P	L3-4 P	L3-4 P	L3-4 P
Control	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	%red						
T1	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	%red						
T2	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	%red						
T3	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	%red						
T4	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	%red						
T5	1						
	2						
	3						
	4						
	5						
	Total						
	Mean						
	%red						

ANNEX 4. MEASUREMENTS AND CONVERSION

MEASUREMENTS AND CONVERSIONS

Volume
1 l = 1000 ml
1 ml = 1000 µl
1 cubic foot = 7.5 gallons = 28 l
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 = 39.7 inches
1 inch = 2.54 cm = 0.0254 m
1 foot = 0.333 yards = 0.3048 m
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 = 2.2 pounds
1 g = 0.035 ounces

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.

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June 2017, Addis Ababa Ethiopia