Ethiopian Public Health Institute (EPHI)

Protocol for evaluation of performance of Indoor Residual Spray chemicals against Anopheles arabiensis in Ethiopia

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March 2017

Addis Ababa, Ethiopia

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Abbreviations

CDC: Center for Disease Control

ELISA: Enzyme-linked Immunosorbent Assay

EPHI: Ethiopian Public Health Institute

HBI: Human Blood Index

HLC: Human Landing Catches

IRS: Indoor Residual Spray

LLINs: Long-lasting Insecticide Nets

MHD: Man-hour Density

MSDS: material safety data sheet

NGOs: Non-governmental Organizations

PCR: Polymerase Chain Reaction

PHC: Primary Health Centre

PHPs: Public Health Products

PSC: Pyrethrum Spray Sheet Collection

PVC: Polyvinyl Chloride

RH: Relative Humidity

WHO: World Health Organization

WHOPES: WHO Pesticide Evaluation Scheme

1 Background

In Ethiopia, malaria is among top public health significant diseases. Almost three-fourths of the total landmass and above half of the total population are at malaria risk (1). According to National Malaria Control Program recent report about 2,174,707 malaria cases and 662 deaths occurs annually (2). Generally, indoor residual spray (IRS) is one of the key elements of malaria vector control and has been in use since the 1950s. IRS is the application of a long-lasting, residual insecticide to potential malaria vector resting surfaces such as internal walls, eaves and ceilings of all houses or structures (including domestic animal shelters) where such vectors might come into contact with the insecticide.

Since 2005, large-scale application of chemical-based malaria vector control including indoor residual spray and long-lasting insecticidal nets (LLINs) in Ethiopia (3). However, appearance and widespread of resistance strains of malaria transmitting vectors becomes a potential threat to eliminating malaria from the country in the upcoming decade. There are local evidences showing the widespread of insecticide resistance to various chemicals used for IRS (4, 5, & 6).

DDT spraying was used since the mid-1960s and discontinued in 2009. DDT resistance was widely distributed in the country and DDT was replaced by deltamethrin. Furthermore, in 2012 Ethiopia switched from deltamethrin to bendiocarb for IRS in response to the observed resistance [4]. Malaria vector control in Ethiopia is targeted mainly against *Anopheles arabiensis* as this species is responsible for transmitting *Plasmodium falciparum* and *P. vivax* (7). IRS application should coincide with the build-up of vector populations just before the onset of the peak transmission season [8] and is conducted in many parts of Ethiopia before the small and big rains usually in February and June (7).

An. arabiensis is the most important malaria vector in Ethiopia (9). It is found in all administrative regions of the country (10). Other malaria vectors in the country include *An.*

pharoensis, *An. funestus*, and *An. nili* (11). Abose *et al*. (12) reported DDT resistance of the primary malaria vector in Arbaminch and Gambella, southern and southwestern Ethiopia. Balkew *et al.* (13) also reported different resistance levels to permethrin and propoxur against the same vector in eastern Ethiopia. Presently unpublished reports also have indicated that the country's primary malaria vector has developed different levels of resistance to all classes of insecticides that are being used for vector control. This calls for generating quality data on insecticides currently in use and new ones to be registered for use in the future.

Monitoring of insecticides is essential to determine the periods that it remains effective in interrupting malaria transmission and schedule when to re-spray (14). The efficacy of IRS could be influenced by different factors such as mosquito susceptibility to insecticide, mosquito behavior (endophilic and endophagic), type of sprayable surfaces, quality of IRS, residual efficacy of an insecticide and community acceptance (15; 16).

In addition, other factors have been reported to contribute to the residual efficacy of an insecticide, such as: the type of insecticide; formulation; applied dose; physical and chemical properties of the sprayed surfaces; and weather conditions (17-19). The insecticide should be sufficiently stable to maintain biological efficacy on treated surfaces over time in order to minimize the number of spraying cycles required to cover the targeted malaria transmission seasons (20). An insecticide for IRS is considered to have adequate residual efficacy when mortality of the exposed mosquitoes is $\geq$ 80% at 24 hours post-exposure (15).

Therefore, there is a critical need to establish evidence based information for the insecticide (s) that would be used as alternatives for the IRS. And also the purpose of this document would be to serve as the modus operandi for the evaluation of the residual activity of alternative insecticides to insecticides currently in use in the country.

2 Rationale and purpose

It has been the responsibility of the Ministry of Agriculture and Natural Resources to register and allow introducing new insecticides for public health use including malaria vector control by the National Malaria Control programme and other insecticides used for household purposes. Since widespread of insecticide resistance and poor quality of chemicals might compromise the intended outcome of vector control there is a need to undertake field evaluation in the local setting. This requires a clear guidance and rigorous scientific approach based on internationally agreed tools applicable and contextualized to the Ethiopian situation. In this regard, EPHI prepared a protocol in 2017 that relied on WHO guidelines (20).

Thus, the main purpose of this protocol is to guide evaluation activities of the performance of IRS chemicals against *An. arabiensis* under laboratory, experimenta hut and field conditions in Ethiopia that help in informed decision of the regulatory and operational use by responsible government institutions.

It is pertinent to assist national vector-borne disease control program, and other relevant agencies, in the evaluation of insecticidal efficacy and monitoring of the susceptibility of chemicals. The trials for evaluation of efficacy of new insecticides (insecticides not yet recommended by WHOPES for use in IRS) are performed in experimental huts (small scale evaluation) and evaluation of WHOPES recommended insecticides are carried out under operational conditions (large scale evaluation). The information will be useful in planning the introduction of new insecticides, replacement of resistant insecticides and making decisions to procure susceptible insecticides under local ecological settings.

The insecticides which are evaluated for the first time (new insecticides), the standard specifications of the compound should be provided by the sponsoring agency. The sponsoring agency should also provide toxicological indices on safety for humans and non-target organisms

especially against domestic pet animals. In other words, the material safety data sheet (MSDS) for the new insecticide should be provided.

This protocol is designed for evaluation and monitoring various IRS insecticide products. The protocol is intended for use by those responsible malaria control bodies. These include national malaria control programme personnel; regional malaria control programme, malaria sentinel site entomology technicians, health facilities, NGOs and relevant international and bilateral agencies and other donors in support of malaria vector control.

3 Laboratory evaluation

Laboratory efficacy of new technical insecticides 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.

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

Duration: 3 months

Objectives

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

3.1 Intrinsic insecticidal activity

Objective

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

This is done by the topical application of an active ingredient to isolate toxicity from confounding effects resulting from insect behavior (20).

Method of testing intrinsic toxicity

- The technical grade insecticide is dissolved in acetone, a highly volatile organic solvent that remains on the insect cuticle only for a short time, to prepare topical solutions. The dosage is expressed in nanograms of active ingredient per mg of body weight of live mosquito.
- Fifty non-blood-fed susceptible female mosquitoes are weighed initially to determine the average live-weight.
- A constant volume of 0.1 µl is added on the pronotum using a pipette. Adding larger volumes should be avoided as it may cause higher mortality due to solvent toxicity.
- After testing with wide range of concentrations, a narrow range of at least five concentrations causing a mortality range from 5% to 99% (preferably 2–3 dosages <50% and 2–3 >50%) should be selected and used per test. A total of 50 susceptible, non-blood-fed, 2–5 day-old female mosquitoes are tested at each concentration.
- The mosquitoes are anaesthetized (using CO_2 for 30 seconds) and placed on a plate under cooling at 4 °C and thereby the anesthesia condition is maintained during the manipulations.
- For the treatment group, two batches of 25 mosquitoes are tested at each concentration of the insecticide.
- Using a suitable applicator, 0.1 µl of the insecticide solution of the required concentration is deposited on the pronotum of the females, running a parallel control of two batches of 25 female mosquitoes treated at 0.1 µl of pure acetone. After dosing, the females are transferred into clean holding cups provided with 10% sugar solution soaked on cotton wool and held for 24 hours at 27 + 2 °C temperature and 80 + 10% RH to record the mortality as a result of topical application.

- The test is repeated three times testing separate batches of reared mosquitoes and the results of the three tests are combined for statistical analysis.
- Whenever the test is repeated fresh insecticide dilutions should be prepared and used.
- Log-dose probit regression (21) is used to analyze the relationship between dose and mortality. LD50 and LD90 and their 95% confidence limits are determined using commercial software. If mortality exceeds 20% in controls, the test is rejected. If mortality in the controls is between 5% and 20%, the treated mortality is corrected to the control mortality using the Abbott's formula as given below:

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

Where

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

Y = percentage mortality in the control.

The probit mortality per log dose regressions for two insecticides could be compared using a parallelism test (20).

3.2 Diagnostic concentration

The diagnostic concentrations are generally used to detect or monitor the presence of resistance in the target vector species to the given insecticide. The diagnostic concentrations recommended by the WHO for each group of vectors are chosen so that exposure for a standard period of 1 hour followed by 24 hours holding can be relied upon to cause 100% mortality of individuals of susceptible strains. To avoid false reporting of resistance in the field (where there is no true resistance), WHO sets the diagnostic concentration at twice the minimum concentration that causes 100% mortality. The prescribed dosage of insecticides (on impregnated papers) and time of exposure for different vectors are given in Table 7.

3.2.1 Preparation of insecticide impregnated papers

- Diagnostic dosage is determined by exposing the target mosquito species to a graded series of dosages of insecticide (technical grade) impregnated on filter-paper.

- Two ml of acetone, and mixed with a non-volatile carrier such as silicon oil (e.g. BDH Dow Corning® 556) or Risella® (Shell) or olive oil (according to the insecticide to be tested) is applied to rectangular pieces of Whatman® No. 1 filter-paper measuring 12 x 15 cm. .
- The carrier oil allows the formation of a stable, thin and homogeneous layer of the insecticide on the filter-paper and also prevents crystallization of active ingredients.
- Since acetone is volatile, the concentration of the insecticide is normally expressed as % of active ingredient (a.i.) per unit volume of carrier oil on the filter-paper.
- Filter-papers are impregnated with 3.6 mg/cm² of the carrier oil, i.e. 648 mg/paper or 0.66 ml/paper for silicon oil (having a density of 0.98). A filter paper impregnated at 1%, contains 6.6 mg of technical insecticide, or 367 mg/m².
- The filter paper is impregnated by pipetting the insecticide solution evenly on to the paper pinned on a cardboard.
- The papers after impregnation are air dried for 24 hours and used for testing. The impregnated paper should not be used more than six times (23).

- The adult susceptibility test (WHO tube test) method as described in 5.11.2.

3.2.2 Determination of diagnostic dosage

Mosquitoes are exposed to graded series of concentrations of the insecticide impregnated on papers as given in 5.11.2. Concentrations should be chosen in such a way that at least one concentration gives 100% mortality, at least two give between 50% and 99% mortality, and at least two give between 5% and 50% mortality.

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

3.3 Residual effect on substrates

This is to identify the target dosages for small scale trial in experiment huts based on residual activity of the sprayed insecticide deposits on different surfaces.

- Residual activity is tested on different pre-fabricated substrates such as mud, dung, brick, thatched, painted, cemented, tin, etc.
- Spraying is done using internationally recognized and precise sprayer for laboratory test called ***Potter sprayer Tower®.***
- The substrates are 40 cm × 40 cm prepared on wooden frames. A minimum of seven replicates per dosage for each substrate are prepared, at least four for bio-assay and three for initial chemical analysis, selected at random. For chemical analysis, three samples from each of the three substrates are used.
- Bio-assays are done in the beginning to determine the lowest concentration causing 100% mortality.
- All the seven replicates for each substrate are sprayed with the insecticide to make a homogenous residual deposit of the desired range of concentrations (2-4 times of the lowest concentration that causes 100% mortality) per unit area using a Potter Spray Tower®, the internationally recognized method for laboratory spraying (25).
- All the treated substrates are stored unsealed under controlled conditions of temperature ($30^oC + 2^oC$), relative humidity (80%), air circulation and ambient light cycles until they are used for testing.
- Residual activity is determined by cone bioassays on four replicates per dosage for each substrate exposing the target vector species for 30 minutes and recording the mortality after 24 hours holding period. After the insecticide application, bio-assays on the treated substrates should be done initially for one week and subsequently at fortnightly/monthly intervals until the mosquito mortality drops below 80%.
- From this assessment, three to five best dosages will be selected for experimental hut evaluation.

3.4 Irritant or excito-repellent properties

One of the important properties of an insecticide is its irritancy. This property needs to be considered during evaluation as it alters the time of the mosquito tarsal contact with the treated surface. Irritancy of an insecticide is studied by releasing mosquitoes in to a WHO specified cone made of polyvinyl chloride (PVC) fixed on an insecticide treated surface. The mouth of the cone is closed with a polyethylene plug. The released mosquitoes in the cone remain in contact with

the insecticide treated surface since they do not generally prefer to rest on PVC cone or polyethylene plug.

- The insecticide irritancy is first assessed using a filter paper impregnated with the technical grade of the given insecticide at the diagnostic dosage as described in section 3.2.2.
- If there is any significant irritancy with the treated filter paper compared to the control (paper impregnated with acetone and silicon oil only), further tests are carried out with appropriate formulations of the insecticide on substrates commonly used for making houses/ shelters (mud, dung, paint, brick, cement, plywood, thatch).
- The selected surfaces are sprayed with the recommended dosage (i.e. the lowest one causing >80% mortality for longer duration) of the insecticide.
- For each test, susceptible, non-blood fed, 2-5 days old female mosquitoes (50 numbers) are individually introduced in to plastic cones.
- After allowing 60 seconds for the mosquitoes to settle down, the time elapsed between 'first landing' and the 'next take off' of the mosquito is recorded as FT.
- Mosquitoes are then grouped by classes of first take off time (0-1 s, >1-2 s, >2-4 s, >4-8s, … >128-256 s) and FT50 and FT95 (the time before 50% and 95% of the mosquitoes take off) are calculated based on cumulative frequencies using probit analysis.
- Mosquitoes that do not take off at least once during the 256 seconds exposure (test period) are discarded.
- An insecticide that is well known for its irritancy (e.g. Permethrin) should be used as a positive control wherever possible.

3.5 Cross resistance

To assess the cross resistance in mosquito vectors that have developed resistance to other insecticides in use under vector control programme, susceptibility tests will be carried out using WHO test kit as described in section 5.11.2.

The susceptibility status of vector species should be categorized as per the WHO criteria: susceptible- 98 to100% mortality, verification required- 80 to 97% mortality, resistant < 80% mortality.

4 Small scale evaluation

Small scale evaluation is carried out in experimental huts and used to evaluate the efficacy and residual effect of chemicals. Those insecticides not recommended by WHOPES are evaluated under experimental huts.

Objectives

General objectives

- To assess the efficacy and residual activity of insecticides against *An. arabiensis.*

Specific objectives

- To measure the efficacy of insecticides in terms of mortality and residual effect.
- To record the ease of application and perceived side-effects by the spray-men during application.

Duration: 6 or more months based on manufactures claim.

4.1 Experimental hut study design

The huts represent common Ethiopian Tukuls and in each hut four types of surfaces (rough mud, smooth mud, painted and dung) are present. The sailing is made of locally available materials and covered by thatches. The radius of the hut is two meters and the height of the wall is two meters with one door. The insecticide treatment is carried out in four houses and two experimental huts sprayed with water serve as a control.

4.2 Safety and precautions in implementation

During the trials, adequate safety precautions and the necessary protective measures should be strictly adhered. Guidelines for the treatment of intoxication and antidote should be available in the trial site and a responsible person should have the access to them. Prior to initiation of any trial, it should be ensured that the experimental huts are completely renovated and cleaned. If the hut is already sprayed with an insecticide, the sprayed surfaces should be replaced and absence of contamination needs to be demonstrated by conducting suitable bioassay tests. The insecticide formulation should safely and correctly be applied in the huts following the WHO guidelines (20).

4.3 Assessment of spray quality

To assess the quality of spraying, three Whatman® filter paper No. 1 (size 10 cm x 10 cm) leveled properly will be struck on the walls of each surface type (one at lower, one at middle and one at upper part) of each experimental hut, before spraying, and removed after complete drying. The papers will be wrapped in aluminum foils and subjected to analysis for insecticide content. The chemical analysis results are combined for each substrate to provide the average concentration of insecticide (in mg/m).

4.4 Cone wall bioassay test

Assessment of the bio-efficacy and residual activities of insecticide sprayed wall surfaces is a longitudinal study aimed at collecting information on monthly basis for six or more month period. Bio-efficacy and decay rate of the insecticides on walls will be measured using standard WHO cone tests [21] in houses on different wall surfaces and each surface will be replicated four times and one unsprayed house will serve as control. To ensure the quality of spraying, cone testes will be carried out one to two days after spraying. Thereafter tests of treated surfaces will be performed on monthly bases post IRS for months.

On a single wall surface three cones will be fixed using fine steel pins at the lower, middle and upper parts. Cones will not be fixed on areas 60 cm above the ground and 60 cm below the roof. Three to five days-old unfed females of *An. arabiensis* of insectary colony will be used. Ten mosquitoes will be gently transferred into each cone and exposed for 30 minutes. At the end of exposure time, the mosquitoes will be transferred into insecticide free holding paper cup for a 24 h holding period and will be supplied with 10% sugar solution. Paper cups will be kept in wooden box or cartoon which will be covered by damp towel to create favorable temperature (27±2) and humidity (70±10). Bioassays are terminated when the mortality is below 80 % in two consecutive bioassays.

4.5 Acceptability of the insecticides by the spray-men

Data will be collected from interviewing spray-men using well structured questionnaires. Ease of application (mixing and spraying) and perceived side effects are recorded.

5 Large-scale field evaluation

Evaluation in this phase is done at the small scale (Company) recommended dosage on a large scale [village(s)] against disease vectors prevalent in the area. Large scale trials are carried out at village level selecting one or more villages. The evaluation should be carried out at least in three eco-epidemiological settings (multi-centric), preferably in two high endemic areas and one low endemic area. Informed consent should be obtained from the human volunteers associated with the evaluation

Objectives

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

5.1 Study site selection

Three study sites are selected based on the history of malaria transmission intensity/ endemicity, IRS usage and effectiveness from the 25 sentinel sites.

5.2 Study design

This study employs a cross-sectional observational study design in purposively selected villages. Selected villages are operational units for the intervention or control arms of the study. Intervention and treatment villages of the study would be comparable in terms of size, human population, proximity to vector breeding sites, LLIN coverage and usage, entomological parameters including vector density, susceptibility to alternative insecticides, etc. Each village are allocated either to intervention or control arms at random in order to minimize the bias attributable to risk factors and to permit a clear demonstration of the effect of the intervention. Considering the flight range of the vector the distance between the intervention and control villages would be at least five kilometers to minimize mosquito infiltration between the intervention and control groups.

The villages can be stratified in pairs (Matched pair designs) and from each pair one village is randomly assigned to the treatment arm and the other to the control arm. Stratified designs are usually preferable to matched pair designs. The number of entomological monitoring sites should be equal in each village. Since houses may vary greatly in their attractiveness to mosquitoes, for practical reasons and consistency, the same entomological sites should be monitored throughout the study (20). House selection for wall bio-assay is based on the availability of different wall types such as mud (rough, smooth), dung, painted and brick. Each insecticide treatment test will be performed in four houses of each wall type on the condition that all wall types are present in each study village.

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

5.3 Ethical considerations

Ethical clearance should be obtained from the EPHI or other appropriate institutions and authorities. This should include the examination of the study protocol, the informed consent form and the trial's information sheet, which will be provided to the study communities.

5.4 Census

In collaboration with the respective Primary Health Centre and District Public Health bureau, census and numbering of all houses in the selected experimental and control villages should be carried out prior to spraying. Census details are recorded in the format as given in Table 1.

Table 1. Record of census of households

Village_____ Sub-centre_____ PHC_____ District_____

State_____ House No. _____ Type of the structure_____

Date of survey_____

S. No.	House No.	Name	Relation	Age/ Gender	Education	Profession	Type of strt	No. rooms	No. of cattle sheds	No. of temporary sheds	Sleeping habit inside/ outside
1.											
2.											
3.											
4.											
5.											
6.											

5.5 Setting the application of insecticides

The IRS is conducted by well trained personnel. They are recruited from the local community and supervised by study team from EPHI.

5.6 Insecticide safety procedures

Insecticides, like drugs, have inherent potential hazards. However, if they are handled and applied according to label specifications, they will be safe and effective. Safety instructions must be followed at all times to avoid potential problems for operators, household residents, pets and domestic animals and the environment. Preparations undertake in households prior to the spraying including: removal of movable household contents specifically water, food, cooking utensils and toys; covering of non-movable contents with plastic sheets; removal of wall coverings and curtains and relocating pets and domestic animals away from the house until sprayed surfaces have dried and the dead insects have been swept up and removed from the floor. Household occupants are instructed to stay outdoors during and for at least 2 h post spraying.

5.6.1 Insecticide poisoning and first-aid measures

Failing to follow correct procedures during spraying operations can result in undesired exposure to insecticides or accidental insecticide poisoning. Below are some of the signs and symptoms of insecticide poisoning:

General – extreme weakness and fatigue;

Skin – irritation, burning, excessive sweating, obvious staining;

Eyes – irritation, burning, excessive running, blurred vision, narrowing or widened pupils;

Digestive system – burning in mouth and throat, excessive salivation, nausea, vomiting, stomach cramps or pains, diarrhoea;

Nervous system – dizziness, confusion, restlessness, headaches, muscle twitching, staggering, slurred speech, fits or convulsions, unconsciousness; and

Respiratory system – breathing with difficulty, wheezing, coughing, chest tightness and pain.

5.6.2 Treatment of insecticide side-effects

Local health units and hospitals should be provided with simple information on the side-effects of insecticides being used and on recommended treatment. In addition, supplies should be checked regarding availability of antidotes. If suspected poisoning occurs the spray operator should seek medical help and show the empty sachet or a product label to a health professional in order to identify the source of poisoning.

The key products or antidotes that should be available for treatment are:

- ✓ topical vitamin E (tocopherol acetate) for skin exposure
- ✓ topical anaesthetic for eye exposure
- ✓ atropine for ingestion exposure
- ✓ diazepam for ingestion exposure
- ✓ phenytoin for ingestion exposure.

5.7 Conducting application of insecticides

IRS alternative chemicals will be sprayed on all unit structures at the recommended doses. Spraying will be done by using a Hudson® X-pert compression sprayer (Hudson Manufacturing Company) with a flat nozzle as recommended for IRS following the national spraying operation guidelines (23) and (24). A distance of 45 cm from the nozzle tip to the surface to be sprayed will be maintained during spraying. At this distance, the width of the swathe at the point of impact is 75 cm. A 5 cm overlap will be maintained between the swathes to make sure that no wall surface will be left without insecticide.

5.8 Quality control

To assess the quality and accuracy of insecticide treatment on indoor surfaces in each house, three pieces of Whatman® filter paper No. 1 (size 10 cm x 10 cm) will be attached on the lower,

middle and upper wall parts in each sprayed house. The filter papers will be suspended away from the surface being sprayed in order not to absorb excessive insecticide by runoff. On the same day after spraying, all the filter paper samples will be collected and packed in aluminium foil and labeled according to the dose, formulation and type of surface and stored in a refrigerator at 4 °C. Six to nine samples for each dose and type of surface will be selected randomly for chemical analysis. The spots where filter papers affixed will be marked with chalk to avoid exact placement of cones on them during subsequent cone bioassay tests. The residents will be advised against washing, painting, plastering or smearing of the walls during the entire study period, as these activities, during efficacy trials, can be detrimental to the residual action of the insecticide.

5.9 Mosquito collection

Mosquitoes will be collected using either all or some of collection methods, including human landing catches (HLC), pyrethrum spray sheet collection (PSC) which will be done with a pyrethrum flit gun or non-residual pyrethroid aerosol, CDC light trap and hand aspiration method as appropriate to the specific objectives of the study every month after spraying. Peak biting hours HLC will be carried out for two nights with two collectors simultaneously stationed indoors and outdoors in two houses per study villages. Catchers will be exchanged for indoor and outdoor collections every hour. For other collecting methods sentinel houses will be selected for each intervention and control villages. Captured mosquitoes will be identified using keys (25) and used to estimate the following entomological parameters:

i. Vector density- estimated by the number of vectors captured/house/ unit time

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

Indoor resting density: Hand catches of resting mosquitoes indoors in the dawn hours are one of the reliable and practical methods of assessing the population density of the vector species and also facilitate estimating biting rates in areas where the vectors are zoophagic and where only small numbers of mosquitoes are obtained per night from human landing catches (HLC). The collections are identified to species and the gonotrophic condition of the female mosquitoes is recorded. In indoor resting catches, if the proportion of half-gravid or gravid mosquitoes is found reduced, it may be an indication of mortality induced by the insecticide or repellency. Indoor

resting collections are also indicative of mosquito biting rates on human if the proportion of mosquitoes feeding on humans is known. The source of blood meal of individual mosquitoes is identified using precipitin (agar-gel diffusion method) or ELISA tests. Using the product of indoor resting collection and the proportion that fed on man (human blood index, HBI), mosquito biting rate on human may be estimated. Hand catches of indoor resting mosquitoes in four to six houses per village at fortnightly/ monthly intervals will give meaningful data on mosquito density, which is expressed as the number per man-hour (man-hour density, MHD).

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

Outdoor resting collections: Outdoor resting mosquitoes could be collected from the natural resting sites such as pit shelters, vegetation, root interstices, tree hollows available in and around the villages. However, searching natural shelters may not be feasible considering the vastness of the area outdoors. Therefore, alternatively, artificial shelters, particularly those which resemble the natural ones and are attractive to the vector species for resting, could be installed and used for the collections (for example, pit traps (pit shelters) dug in the ground).

Such collections may provide information on outdoor resting behaviour if the vector commonly rests outdoors or is driven outdoors by the repellent effect of the insecticide. Four to six shelters may be installed per village. The shelters should preferably be installed under shade and in such a way that they do no face the direction of sunrise. Mosquito collections are carried out in the morning hours and the density is expressed as number collected per shelter or per man-hour.

Mosquito landing collections on human (HLC): This may be done if feasible and on obtaining necessary clearance from human ethics committee. The density of the vector species can also be monitored by conducting HLC that gives the number of landing mosquitoes per person per night. All night (dusk to dawn) mosquito landing collections should be made in one house in each

treatment and control village at fortnightly intervals. Collections are required both inside and outside the houses to assess biting rate indoors and outdoors (endo- and exophagy). Persons volunteering to be baits should be informed about the experiment. Informed consent of the volunteers involved in the study should be obtained prior to the collections. The human volunteers may lie down or on a cot and can sleep as per their normal sleeping practice, exposing legs up to knees. The insect collectors, who will be catching the mosquitoes landing on the bait, are rotated every four hours to avoid bias and slackness.

The sampling errors caused by variation in catcher efficiency or attractiveness may be reduced by increasing the number of capture sites per cluster. Hourly mosquito collections should be recorded in the format given in Table 3. Results are expressed as number of vectors landing per human bait per night. If the collections are restricted to the hours of peak biting of the vector species, the results are expressed as number of mosquitoes landing per bait per hour. The results would provide information on biting periodicity and feeding habits of the vector species in the study areas.

Table 2. Mosquito landing collection on human (HLC)

Village_____ Sub-centre_____ PHC_____ District _____

State_____ Date of collection_____ Insecticide & Dose_____ Spray Round_____ Temperature: Min_____ Max _____Relative humidity: Min _____Max _____

Time (Hrs)	***An. gambiae*** M UF* BF SG G T NP P	***An. pharonsis***		*An. custouni*		**Culicines**	
		M	F			F	M

1800 1900 2000 2100 2200 2300 2400 0100 0200 0300 0400 0500 0600							
Total							
M = Males; UF = Unfed females; BF = Blood fed females; SG = Semi-gravid females; G = Gravid females; P = Parous; NP = Nulliparous; T = Total dissected; *Unfed mosquitoes should be dissected for parity							

Mosquito landing collections on animals: This may be done if feasible and on obtaining necessary clearance from animal ethics committee. In areas, where the vectors are mainly zoophagic or present at low densities, HLC results in low capture rates and poor catcher efficiency. Therefore, to measure more accurately the abundance of the zoophagic vector(s) in a sprayed cluster, collections of landing mosquitoes on domestic animals (usually cattle) are made at fortnightly intervals. Landing collections on a cattle tied to a pole are made from dusk to dawn. Data should be recorded in the format given in Table 3. Results are expressed as number of vectors landing per animal bait per night or number of mosquitoes per bait per hour, if the

collections are restricted to the hours of peak biting. This will provide information on biting rhythm and feeding habits of the vector species in the area.

Light trap catches: In areas, where there is a correlation between the light trap (hung at the side of occupied untreated nets) catches and HLC, light trap catches can replace HLC. Light trap Mosquito collection using light traps is relatively easier and much less labour-intensive than HLC. Therefore, light traps (CDC light traps or its modified versions) could be a reliable alternative that overcome the ethical constraints and remove human error (while mosquito collection) associated with HLC. The traps are set indoors (human dwellings or animal sheds) as well as outdoors during dusk hours at fortnightly intervals in both treated and control villages. The next morning, the trapped mosquitoes are collected, identified to species and recorded in the format given in Table 3.

Table 3. Light trap collection

Village_____ Date of collection _____ Insecticide & Dosage _____ _____ Spray Round_____ Collection site No & Type _____ Temperature: Min_____ Max ____ Relative humidity: Min ____ Max ____

Trap	**Collection**	***An. arabiensis***	**Culex mosquitoes**
No. site	No and type	M UF* BF SG G T NP P	M F
1 2 3			
M= Males; F= Females; UF= Unfed females ; BF= blood-fed females, SG= Semi-gravid females; G= Gravid females; P = Parous; NP= Nulliparous; T= Total dissected; * Unfed mosquitoes should be dissected for parity			

Pyrethrum Spray sheet collection

Pyrethrum Spray Catches (PSC) collects indoor resting mosquitoes by spraying an insecticide (pyrethrum) into the space. White cloth is spread on the evening on the entire floor of the house

before inhabitants retire to bed. Early morning, before the inhabitants resume their regular activities, the dead and morbid mosquitoes lying on the floor sheet are picked up with forceps and scored. Collections are made on a white sheet following knock-down. Other dead insects lying on the floor should be separately collected and stored for monitoring. The inhabitants of the trial houses are asked not to physically damage the knocked-down mosquitoes. Precautions should be taken to protect the knocked-down mosquitoes from scavengers such as ants. These mosquitoes should be identified to species and abdominal (gonotrophic) condition recorded in the format given in Table 4.

Table 4. Floor sheet collection

Village_____ HC _____ District_____ State_____

Date of collection_____ Insecticide & Dose_____ Spray Round_____
Type of structure:

Mud/cement/brick/Thatch/ stone/_____Temperature: Min___ Max_____ Relative humidity: Min __ Max____

House code	Species	Males	Females				Total no (dead + morbid)
			UF*	BF	SG	G	
1.							
2.							
3.							
4.							
5.							
6.							
7.							
8.							
9.							
10.							

UF = Unfed; BF = Blood fed; SG = Semi-gravid; G = Gravid; *: Unfed mosquitoes should be dissected for parity

ii. Vector longevity

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

iii. Infectivity rate

Vector mosquitoes obtained from HLC and indoor resting hand catches are dissected out in 0.6% saline to examine their mid-gut for the presence of oocysts and salivary glands for sporozoites using microscopy (26) or ELISA (27; 28) or PCR (29). If the IRS is effective, only a few mosquitoes would survive the time required for sporozoites to develop and mature, and therefore a marked reduction of the sporozoite rate is expected. In areas, where the infection/ infectivity rate is very low, pooled samples (pool size needs to be standardized) can be used for enzyme-linked immunosorbent assay (ELISA) test with no loss of sensitivity. By testing pooled samples, the numbers of tested mosquitoes could be increased to thousands which is necessary to conclude that there is a significant reduction after IRS and also to make meaningful comparisons between study arms. Results are recorded in a format as given in Table 5. Data should be represented for each insecticide and dosage separately.

Table 5. Vector infection and infectivity rates

Village_____ Sub-centre_____ PHC_____ District_____

Date of collection _____ Insecticide & Dose_____

Species	No. dissected	Oocyst positive	Sporozoite positive	Oocyst rate	Sporozoite rate	ELISA positive	PCR positive
1. 2. 3.							

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

iv. Human blood index

The Human Blood Index (HBI) represents the proportion of blood meals derived from humans by mosquito vectors. It may be used to estimate the human biting habit, an important component of vectorial capacity, as a proxy measure of malaria transmission (30; 31). HBI of the collected blood-fed mosquitoes will be determined using a direct ELISA following the method of (32) and modified by Loyola et al. (33) is used to identify the source of the blood meal.

The crude HBI was calculated using all the mosquitoes collected in the denominator. For the adjusted HBI, vectors with unidentified blood meal sources were excluded from the calculation. Human blood indices is calculated for each species. Mixed blood meals are included in the HBI calculation. For the statistical analysis, the SAS 9.1 software package is can be used, and the Fisher's exact test is performed. It can be also determined by employing a polymerase chain reaction (PCR) assay.

5.10 Residual activity of insecticide/cone wall bioassay test

Assessment of the bio-efficacy and residual activities of insecticide sprayed wall surfaces is a longitudinal study aimed at collecting information on monthly basis until the insecticide gives ≤80% mortality of exposed mosquitoes. Bio-efficacy and decay rate of the insecticides on walls will be measured using standard WHO cone tests (20) in houses with different wall surfaces and each surface will be replicated thrice for each chemical and three unsprayed houses for each surface will serve as controls. To ensure the quality of spraying, cone testes will be carried out one to two days after spraying. Thereafter tests of treated surfaces will be performed on monthly bases post IRS for months.

In a single house three cones will be fixed using fine steel pins on the walls at the lower, middle and upper parts. Cones will not be fixed on areas 60 cm above the ground and 60 cm below the roof. Three to five days-old unfed females of *An. arabiensis* from EPHI insectary will be used. Ten mosquitoes will be gently transferred into each cone and exposed for 30 minutes. At the

end of exposure time, the mosquitoes will be transferred into insecticide free holding paper cup for a 24 h holding period and will be supplied with 10% sugar solution. Paper cups will be kept in wooden box which will be covered by damp towel to create favorable temperature (27±2) and humidity (70±10).

Mortality counts will be taken after 24 h post exposure. Mosquitoes will be classified as dead if they are immobile or unable to stand or fly in a coordinated way. When average mortality of the controls is between 5% and 20%, test mortality will be corrected using Abbot's formula. But if control mortality is above 20%, the results will be discarded and the tests will be repeated.

Table 6. Cone bioassays for residual activity

Village _____ Sub-centre_____ PHC _____
District _____ Date of bioassay_____ Insecticide & Dosage _____ _____Temperature: Exposure _____ Holding _____
Humidity _____Date of last spray and round_____ Type of surface: Mud (rough, smooth, dung)/cement/brick/thatch _____

Test specie _____ Lab/F1/Field collected _____

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

5.11 Mosquito rearing and insecticide susceptibility test

5.11.1 Mosquito rearing

Anopheles larvae and pupae will be collected from selected sites and kept for rearing to adult. Adults will be kept in big cages and ready for further insecticide susceptibility testes.

5.11.2 Insecticide susceptibility test

Susceptibility test is conducted using the WHO test kit and method (20). The test kit and papers impregnated with insecticides at the WHO recommended diagnostic dosage could be obtained on payment from by the Vector Control Research Unit, Universiti Sains Malaysia, Penang, Malaysia. It is recommended that initial susceptibility tests using discriminating concentrations be performed on adult females aged 3–5 days that are nonblood fed (i.e. sugar fed and starved for about 6 hours). Larval collections should ideally be made from a number of different breeding sites to avoid sampling individuals from single egg batches, which could result in a high proportion of siblings in the test population.

- Female *An. gambiae* s.l. will be morphologically (25) selected under dissecting microscope.
- The investigator puts on gloves. Six sheets of clean white paper (12 × 15 cm), rolled into a cylinder shape, are inserted into six holding tubes (with the green dot), one per tube, and fastened into position against the wall of the tube with a steel spring wire clip. The slide unit is attached to the tubes at the other end.
- Ideally, 120–150 active female mosquitoes are aspirated (in batches) from a mosquito cage into the six green-dotted holding tubes through the filling hole in the slide, to give six replicate samples of 20–25 mosquitoes per tube.
- Once the mosquitoes have been transferred, the slide unit is closed and the holding tubes set in an upright position for 1 hour. At the end of this time, any moribund mosquitoes (i.e. those unable to fly) and dead mosquitoes are removed.
- The investigator inserts one oil-treated paper (the control) into each of two yellow-dotted tubes, ensuring that the label of the paper is visible on the outside of the tube. The paper is fastened with a copper clip and the tube closed with a screw cap.
- Four exposure tubes with red dots are prepared in much the same way as the yellow-dotted tubes. Each of the four red-dotted exposure tubes is lined with a sheet of insecticide-impregnated paper such that print label is visible on the outside. Each paper is then fastened into its position against the wall with a copper spring-wire clip and the tube is closed with a screw cap.
- The empty exposure tubes are attached to the vacant position on the slides and, with the slide unit open, the mosquitoes are blown gently into the exposure tubes. Once all the

mosquitoes are in the exposure tubes, the slide unit is closed (usually a cotton wool plug is inserted into the hole to lock the slide) and the holding tubes are detached and set aside. The investigator now removes the gloves.

- Mosquitoes are kept in the exposure tubes, which are set in a vertical position with the mesh-screen end uppermost, for a period of 1 hour (unless otherwise specified). The tubes are placed in an area of reduced lighting or covered with cardboard discs to reduce light intensity and to discourage test mosquitoes from resting on the mesh-screen lid.
- At the end of the 1-hour exposure period (or longer for certain compounds, as outlined in Table 8), the mosquitoes are transferred back to the holding tubes.
- The exposure tubes are detached from the slide units. A pad of a cotton wool soaked in 10% sugar water is placed on the mesh-screen end of the holding tubes.
- Mosquitoes are maintained in the holding tubes for 24 hours (or longer for slow-acting compounds). During this time, it is important to keep the holding tubes in a shady, sheltered place in the laboratory or in a chamber maintained at 27 °C ± 2 °C temperature and 75% ± 10% relative humidity. Temperature and humidity should be recorded during the recovery period.
- At the end of recovery period (i.e. 24 hours post-exposure or longer for slow-acting compounds), the number of dead mosquitoes is counted and recorded. An adult mosquito is considered to be alive if it is able to fly, regardless of the number of legs remaining. Any knocked down mosquitoes, whether or not they have lost legs or wings, are considered moribund and are counted as dead. A mosquito is classified as dead or knocked down if it is immobile or unable to stand or take off.
- When average mortality of the controls is between 5% and 20%, test mortality will be corrected using Abbot's formula (34). But if control mortality is above 20%, the results will be discarded and the tests will be repeated.
- $\text{Corrected mortality (\%)} = \frac{(\%\ \text{Test mortality} - \%\ \text{Control mortality})}{(100 - \%\ \text{Control mortality})} \times 100$

Table 7. Diagnostic doses of different insecticides for different Anopheles mosquito vectors

Insecticides	**Diagnostic doses**
Organochlorine	
DDT	4% 1 h
Organophosphate	
Malathion 5% 1 h	5% 1 h
Primiphos-methyl	0.25% 1h
Fenitrothion	1% 2 h
Carbamates	
Propoxur	0.1% 1 h
Bendiocarb	0.1% 1 h
Carbosulfan	0.4% 1h
Pyrethroids	
Deltamethrin	0.05% 1 h
Alpha-cypermethrin	0.05% 1h
Cyfluthrin	0.15% 1 h
Lambdacyhalothrin	0.05% 1 h
Permethrin	0.75% 1 h
Etofenprox	0.5% 1 h

Table 8. Insecticide susceptibility test (WHO tube method)

Locality:_____ Site of larvae and pupae collection/Village: _____ GPS position UTM_X _____ UTM_Y _____ Species: _____ Number exposed:_____ Test Date_____ Insecticide:_____ Date impregnation: _____ Expiry date : _____ Number of times this paper is used: _____ Storage conditions: Room temp ☐ Refrigerated ☐Temperature Start: _____End: _____

Holding: _____ RH Start:_____ End:_____ Holding:_____ Exposure time: _____ Minutes: _____

Time	Knockdown in test replicates				Average	Knockdown in control replicates	
	I	II	III	IV		I	II
10							
20							
30							

40							
50							
60							
70							
80							
No. dead after 24 h							
No. alive							
% mortality							
%Corrected mortality							

The interpretation of test results will be done based on standard procedure as recommended by WHO (22). The procedure categorizes mosquito into susceptible if mortality rate is 98–100%, and suggests the possibility of resistance in the vector population and must be confirmed if mortality rate is 90-97% and confirmation of the existence of resistant genes in the test population with additional bioassays may not be necessary if mortality rate is less than 90%.

The quality of the insecticide impregnated papers will be assessed using the susceptible colony of *An. arabiensis* strain from EPHI insectary using WHO test kit [20]. Members of the *An. gambiae* s.l. species complex will be identified using allele specific PCR [35].

5.12 Acceptability of the insecticide by the community

Baseline data will be collected from a random sample of households within intervention villages at the start and end of the study using well-structured questionnaires. Together with the insecticides impact on the target vector species, data for factors that can limit acceptability of the insecticide under consideration, including visible insecticide stains on walls, an unpleasant odor, or skin and nasal irritation would be collected. Ease of application (mixing and spraying) by the spraying operators and impacts on other household insects including nuisances will also be recorded.

5.13 Data analysis

Collected entomological data will be entered into appropriate statistical software for the analysis. Proportion of entomological data (parous and sporozoite rates and bioassay mortality) will be analyzed using logistic regression analysis while numeric entomological data, including mosquito resting density, HLC or CDC light trap catches are going to be analyzed using Poisson regression or transformed using logs to a normal distribution before applying analysis of variance.

6 Data Submission

- Upon successful registration of house in the study area, the data will sent to the research staff enrolling the houses using hard copy format, along with the form due dates at the EPHI. These calendars will be updated as the study proceeds to reflect data that have been received, reply deadlines for queries about unclear data, deadlines for follow-up reports of adverse events, or changes in the protocol that change the data being collected or the timeframe. Updated calendars for each participant can be obtained through mail communication from the PIs.
- The investigative site is required to submit data according to protocol as detailed on each participant's calendar, as long as the IRS evaluation is designated as open/alive or until the study is terminated. The IRS evaluation is closed when all data have been received; reviewed and no outstanding data query exists for the IRS evaluation.
- Once data entry of a form is complete, and the summary form reviewed for completeness and accuracy, No further direct revision of the submitted data is allowed after this point.

7 Data Security

Even though evaluation of IRS insecticides data does not need data security issue like human case research's; for the sake of PIs and Co- investigators ownership of the data, the data of this project released to the web site and other partners under the permission of the PIs, the Co-PI and Co- investigators.

8 Electronic Data Management

Data received from the filed-based forms are stamped with the date and time of receipt by the nearest heath facility. Then the data will enter into the database. The Data checked for accuracy and completeness using Microsoft excel. If checks at EPHI detect missing or problematic data, the EPHI sends a Request for Information (query letter) to the site investigator specifying the problem and requesting clarification. The EPHI updates the participant's data submission calendar with the due date for the site investigator's response.

9 Data Safety and monitoring plan

Describes how the study investigators plan to oversee research subject safety and how adverse events will be characterized and reported. The intensity and frequency of monitoring should be tailored to fit the expected risk level, complexity, and size of the particular study

10 Study population

The study populations for this project are malaria vectors in Ethiopia (i.e *Anopheles gambiae* complex), even though, depending of entomological data collected the study population could shift to only female mosquitoes; in the case of assessing parity.

11 Inclusion Criteria

- Anopheles gambiae complex.
- Female anopheles mosquitoes (for the analysis of vector longevity , infectivity rate, and HBI)
- Unfed and un gravid female mosquito(for the analysis of vector longevity)

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13 Annex

Annex 1: Assessment of perceived benefits, side-effects 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 these diseases? _____ _____

9. If so, how _____ Indigenous _____ Commercial _____

10. Are you aware whether something was sprayed in your house?_____ If yes, when and why _____

11. Generally how many people sleep in the sprayed rooms(s)? _____ _____

12. Do you sleep in sprayed room? _____

13. How does it smell?_____

14. Do the sleepers feel suffocated?_____

15. Have you allowed spraying in all rooms?: ___ If no, reasons _____ _____

16. Does the insecticide leave stains on walls? _____

17. Any fear of poisoning:_____

18. Observations/perceptions of the effect of insecticide

- on mosquito bites
- on bed bugs
- on head lice
- on body lice
- on domestic animals

- any other

19. Do you agree to use insecticide spray in future? YES/NO

Reasons

_____

Signature or LTI of inhabitant Signature of Interviewer

Place/Date:_____

(This format should be translated into respective local language(s) in the study area and

provided to the householder and read to him. A copy of the signed consent form should be given to the householder).

Annex 3: Safety precautions

The following safety precautions should be taken:

- ✓ Read the label carefully and understand the directions for preparing and applying the insecticides, as well as the precautions listed;
- ✓ Follow the directions and precautions exactly;
- ✓ Know the first-aid measures and antidotes for the insecticides being used;
- ✓ Use protective clothing while handling and spraying insecticides;
- ✓ Mix insecticides in a well-ventilated area, preferably outdoors;
- ✓ Rinse container for liquid insecticides properly (see below);
- ✓ Make sure that the spray equipment does not leak and check all joints regularly;
- ✓ Avoid skin contact;
- ✓ Use dedicated equipment for measuring, mixing and transferring pesticides;
- ✓ Use pre-packaged insecticides with the appropriate quantity of water in the sprayer;
- ✓ Ensure the sprayer is depressurized before opening the lid;
- ✓ do not eat, drink, smoke or use mobile phones while handling and spraying insecticides;
- ✓ Wash hands and face with soap and water after spraying and before eating, smoking or drinking;
- ✓ Shower or bathe at the end of every work-day and change into clean clothes;
- ✓ Wash overalls and other protective clothing at the end of each work day in soap and water and keep them apart from the rest of the family's clothes;
- ✓ Change clothes immediately if they become contaminated with insecticides;
- ✓ Keep two sets of protective clothing in different colouring to avoid using the same uniform as the previous day. In this way, it is always possible to use one set while the other is being washed;
- ✓ Do not clear blocked spray nozzles by blowing with the mouth; and
- ✓ Inform the supervisor immediately if feeling unwell.