PHASE 1- LABORATORY TESTS

SOP Title 1- Intrinsic Toxicity Testing

Objective

The objective of this test is to determine the intrinsic activity of the insecticide. This is done via topical application to the mosquito to isolate toxicity from confounding effects resulting from insect behavior or variable dosing. A minimum of five concentrations are required covering a range of mortality from 10% to 90%. Fifty insecticide susceptible, non-blood-fed, 2–5 day-old female mosquitoes are treated at each concentration and 50 treated with acetone alone which serve as negative controls. A minimum of three replications from separate cohorts are required for each test concentration (5 doses x 1 control x 50 mosquitoes and three replicates = 900 mosquitoes). Log Dose Probit analysis is then used to determine the Lethal Dosage (LD) of the experimental insecticide for 50% and 90% mortality (LD50 and LD90).

A batch of mosquitoes (the number of mosquitoes being dependent on the aptitude of the staff member) is anaesthetized with CO_2 for 15–30 seconds and placed on a plate cooled to 4 °C to maintain anesthesia. A volume of 0.1µl of insecticide solution is deposited on the pronotum. After dosing, the mosquitoes are returned to the clean holding cups, provided with 10% sugar solution on cotton wool and held for 24 hours at 27 ± 2 C temperature and 80 ± 10% RH. Temperate species may have different environmental requirements.

Knock down of mosquitoes is assessed at 60 min, and mortality is assessed at 24 hours. A mosquito is recorded as being alive if it can both stand upright and fly in a coordinated manner. A mosquito is recorded as being moribund if it cannot stand (e.g. has one or two legs), cannot fly in a coordinated manner or takes off briefly but falls immediately. A mosquito is recorded as being dead if it is immobile, cannot stand or shows no signs of life. If control mortality is <10%, the results for that day should be adjusted by Abbott's formula. If the control mortality is >10%, the results for that day are considered invalid and should be discarded.

Alongside the blank control the testing of a positive control, of a standard insecticide, is encouraged. The studies are performed against well-characterized laboratory reared mosquitoes. Where possible, all three mosquito genera (Anopheles, Aedes and Culex) should be tested. Finding the correct dose range can be difficult. Therefore, it is recommended to start with a lesser number of test mosquitoes and a large series of doses to find the appropriate range.

Solutions for topical applications are prepared by dissolving technical grade insecticide in acetone, a highly volatile organic solvent, which has the advantage of remaining on the insect cuticle for only a short period of time. The doses used in topical application are typically expressed in nanograms of active ingredient per mg of body weight of live mosquito.

Procedure intrinsic insecticidal activity

Equipment List

- A minimum of 900 non-blood-fed, 2–5 day-old female mosquitoes
- Chill table
- Micro Pipettes
- Paper towels and or filter papers

- Forceps
- Magnifying light
- Aspirator
- Holding cups, labels and pens
- Cotton wool pads, 10% sugar water
- Scales, dilution glass wear, active ingredient and diluent
- Amber vials
- CO_2, a sealed box with a sealed pipe entry from a CO_2 canister or dry ice. A separator in the box provides a uniform distribution of CO_2 and protection from freezing.
- Gloves, lab coat, protective glasses
- Bench cover
- Biohazard bags and disposal options

Preparation of test system

1. Calculate average live weight of the target species by anesthetizing and weighing 50 non-blood-fed susceptible female mosquitoes.
2. Label holding cups to clearly identify each experimental compound, dose, species and replicate.
3. Aspirate mosquitoes into the labeled paper holding cups. The number per cup depends on how many the operator can treat in one run. Catch up an appropriate number of cages to provide the required 50 mosquitoes per dose.
4. Choose female mosquitoes that are fit, appropriately sized, and able to fly consistently. [Parameters for the assessment of mosquito fitness (weight, wing length) must be defined by each test facility based on their knowledge of the species/strains of mosquito they will use for laboratory studies. These parameters, and the method in which they will be assessed, must be described in a SOP.]
5. Put cups in a humidity and temperature controlled holding room set at 27 ± 2 C temperature and 80 ± 10% RH until the test room and materials are prepared.
6. Place a clean bench guard on top of the bench.

Dilutions

1. Put on lab coat, gloves and protective glasses. If possible work in a fume cupboard- turn on air.
2. Prepare the solutions and place each dose in a labeled amber glass container with a chemically resistant lid. Some compounds can be sensitive to light (amber glass) and acetone is highly volatile so the lid reduces evaporative loss of the diluent.
3. Only use glass measuring devices and holding jars because most pesticides adsorb to plastics.

Exposure

1. Ensure the equipment is prepared and clean.
2. Put on lab coat, gloves and protective glasses.
3. Turn on the chill table and allow it to cool to 4°C. Have paper towels or filter paper available to keep moisture off the surface of the chill table: the mosquitoes will stick to the surface if it is not kept dry. In addition, the towel will reduce contamination of the chill table surface; change with every treatment or when wet (Fig 1).
4. Prepare the knockdown box: either connect the CO_2 canister to the box or place the dry ice inside an ice box. Place perforated separator to allow the CO_2 to be distributed evenly to the holding cups and to protect mosquitoes from freezing on the dry ice (Fig Two).

5. A constant volume of 0.1 µl should be delivered to the mosquito pronotum using a calibrated pipettor. The chemical should be applied with the lowest dose first and proceed with increasing concentrations. Change the pipette tip with each treatment (Fig 3).
6. Control cages should be exposed to acetone alone at the beginning of each dose range treatment.
7. Once dosed, the mosquitoes are returned to the original labeled holding cup, provided with 10% sugar solution on cotton wool and held for 24 hours at 27 + 2 C temperature and 80 + 10% RH.

Post Exposure

1. Decontaminate all insecticide-contaminated material according to the specific active ingredient decontamination instructions.
2. Store or dispose of the chemicals as per the instructions from the manufacturer. [The method for the storage and disposal of chemical waste must be described in a separate SOP. This method must conform with national regulations on chemical disposal]
3. Dispose of bench guard paper towels and spent pipette tips in a biohazard bag.

Recording knock down and mortality

1. Record knockdown 60 minutes after treatment application. A mosquito is classified as knocked down if it cannot stand (e.g. has one or two legs), lies on its back moving legs and wings but unable to take off, cannot fly in a coordinated manner or takes off briefly but falls immediately.
2. Record mortality 24 hours after exposure. A mosquito is classified as dead if it is immobile, cannot stand or shows no signs of life. If control mortality is >10%, the test must be repeated.

SOP Title 2- Wind Tunnel Testing

Objective

The aim of this study is to determine the lethal concentration (LC) of the insecticide applied as a space spray (aerosol droplets 15 ± 5 µm). To enable repeatable and comparable testing, a wind tunnel system has been developed that exposes the test mosquitoes to a specified quantity of test compound under controlled environmental conditions. Mosquitoes are aspirated into a fine mesh cage, the test compound is released down a small wind tunnel, penetrating the cage and exposing the mosquitoes to a set volume of space spray. The most important aspects of the wind tunnel design to recreate are the wind speed, the mesh of the cage, the droplet size and volume of spray material to which the test mosquitoes are exposed.

The Wind Tunnel consists of a tube (15 cm in internal diameter metal duct) through which a column of air moves at 3 m/s. Air movement is achieved via a fan at the far end of the tube. The spray cage is a cylindrical screen cage (mesh openings 1.2 x 1.6 mm and 0.28 mm diameter wire) made to fit the interior measurements of the wind tunnel. The cage is inserted into an opening 1 m from the wind tunnel entrance; a flexible clear plastic sheet is used to close the opening. The technical insecticide in an acetone solution (1 - 0.5 ml total volume) is atomized through a nozzle to produce droplets with a Volume Median Diameter (Dv0.5) of 15 ± 5 µm at the position of the cage. The Dv0.5 is the diameter which divides the volume of the spray into two equal halves. The nozzle is a reservoir twin fluid venturi nozzle. The orifice can be large and the pressure low because the atomization is through volatilization of the acetone carrier. This simple nozzle design allows minute quantities to be sprayed in entirety with no test compound left in a dead space. This wind tunnel is only suitable for ULV applications and without special nozzle adjustments would not be suitable for low volume (LV) thermal fog formulations.

A total of 50 insecticide susceptible, non-blood-fed, 2–5 day-old female mosquitoes are used at each concentration, with at least five concentrations covering a range of mortality from 10% to 90%. The 50 mosquitoes are separated into duplicate cages. A minimum of three replications from separate cohorts of mosquitoes are required for each test concentration (5 doses x 1 control x 50 mosquitoes and three replicates = 900 mosquitoes). Ideally, three people should be available to work the wind tunnel. One person to insert and remove the spray cage, one person to spray the chemical and the other to transfer the dosed mosquitoes into clean holding cages. Alongside the blank control the testing of a positive control, of a standard insecticide, is encouraged. The studies are performed against well characterized laboratory reared mosquitoes, where possible all three mosquito genera (Anopheles, Aedes and Culex) should be tested. Finding the correct dose range can be difficult it is recommended to start with a lesser number of test mosquitoes and a large series of doses to find the appropriate range.

Procedure intrinsic insecticidal activity

Equipment list

- A minimum of 900 non-blood-fed, 2–5 day-old female mosquitoes
- Wind tunnel (extraction fan, metal duck tubing, nozzle and nitrogen canister).
- Aspirator
- Spray cages
- Holding cups, labels and pens
- Cottons, 10% sugar water

- Scales, dilution glass wear,
- Active ingredient and diluent
- Amber vials
- CO_2, a sealed box with a sealed pipe entry from a CO_2 canister or dry ice. A separator in the box provides a uniform distribution of CO_2 and protection from freezing.
- Gloves, lab coat, protective glasses
- Bench cover
- Biohazard bags and disposal options

Preparation of test system

1. Aspirate 25 mosquitoes into the spray cage labeled with an appropriate annotation for each experimental compound, dose, species, replicate and sample number. For efficiency sake we let this label follow the mosquitoes from the spray cages to the holding cups, maintaining a precise chain of custody.
2. Choose female mosquitoes that are fit, appropriately sized, and able to fly consistently. [Parameters for the assessment of mosquito fitness (weight, wing length) must be defined by each test facility based on their knowledge of the species/strains of mosquito they will use for laboratory studies. These parameters, and the method in which they will be assessed, must be described in a SOP.]
3. Put spray cages into humidity and temperature controlled room held at 27 + 2 C temperature and 80 + 10% RH until the test room and materials are prepared.
4. An hour before the acclimation period, switch on the humidifier and heater in the wind tunnel room as necessary to 27 ±- 2°C and 75 ± 10%.

Dilutions

1. Put on lab coat, gloves and protective glasses. If possible work in a fume cupboard, turn on air.
2. Prepare the solutions and place each dose in a labeled amber glass container with a chemically resistant lid. Some compounds can be sensitive to light (amber glass) and acetone is highly volatile so the lid reduces evaporative loss of the diluent.
3. Only use glass measuring devices and holding jars because most pesticides adsorb to plastics.

Wind Tunnel Operation

1. Transfer mosquitoes to the test room in the spray cages and hold for 1h acclimatization before testing. Acclimation can exceed 1 hour but should not be less than 1 hour. Remove the glucose-soaked cotton wool from the cups 1 hour before exposure.
2. Record the temperature, humidity, logger ID number, and time acclimation started on the form.
3. Place a clean bench guard on all surfaces, and fix with masking tape
4. Put on lab coat, gloves and protective glasses
5. Record the temperature, humidity, logger ID number, and time of the exposure period on the form at the beginning and end of the session.
6. Begin the tests with the control cage (applying acetone alone) and then with the lowest dose. Proceed with increasing concentrations, following protocol.

7. Place the spray cage in the wind tunnel and close the door.
8. The wind tunnel employs a twin fluid nozzle- turn the air on. Ensure that the air is flowing through the nozzle before the chemical is added to the gravity feed reservoir.
9. Dispense (0.5-1.0 ml) of the test compound into the reservoir and let the chemical atomize and flow down the wind tunnel.
10. Leave the spray cage for 5 seconds to ensure the all chemical has cleared the cage.
11. Remove the spray cage and take to a clean bench. Lightly anaesthetize mosquitoes with CO_2, place in clean holding cups, provide with 10% sugar solution on cotton wool and hold for 24 hours at 27 + 2 C temperature and 80 + 10% RH.
12. Clean the nozzle with a 1 ml spray of acetone between each series of concentrations.

Post Exposure

1. Decontaminate all insecticide-contaminated material according to the specific active ingredient decontamination instructions. [Method for decontamination to be described in a separate SOP]
2. Store or dispose of the chemicals as per the instructions from the manufacturer. [The method fo the storage and disposal of chemical waste must be described in a separate SOP. This method must conform with national regulations on chemical disposal]
3. Dispose of bench guard paper towels and spent pipette tips in a biohazard bag.

Recording knock down and mortality

1. Record knockdown 60 minutes after exposure. A mosquito is classified as knocked down if it cannot stand (e.g. has one or two legs), lies on its back moving legs and wings but is unable to take off, cannot fly in a coordinated manner or takes off briefly but falls immediately.
2. Record mortality 24 hours after exposure. A mosquito is classified as dead if it is immobile, cannot stand or shows no signs of life. If control mortality is <10%, the test must be repeated.

Figure 1 The chill table with magnifying light paper towel to stop condensation and prevent contamination of the table top, the micro pipettes and forceps for application of the compound and manipulation of the test mosquitoes

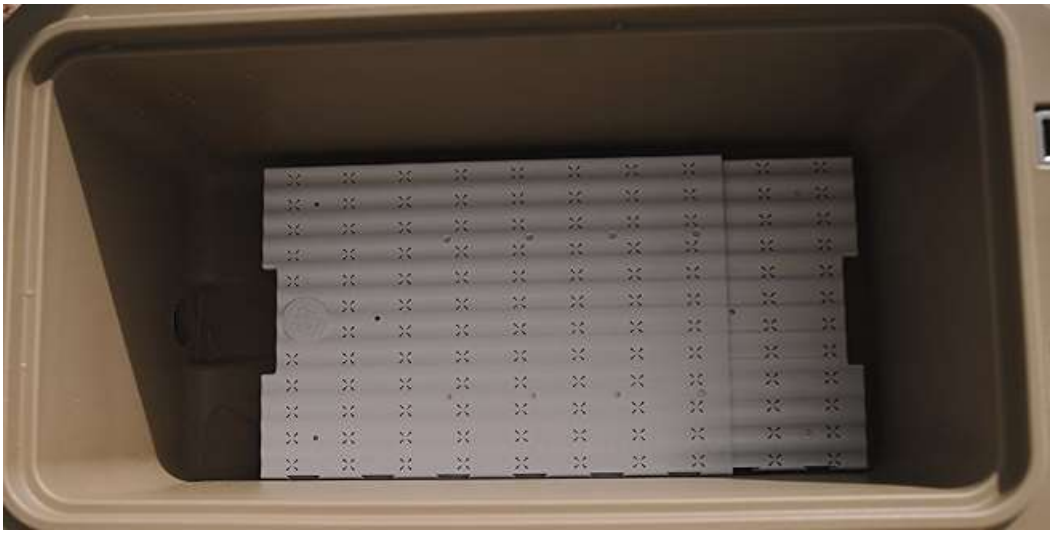

Figure 2 Cooler separator to prevent the mosquitoes from freezing from direct contact with dry ice and aids uniform distribution of the CO_2

Figure 3 The dose of 0.1 µl being placed directly onto the pronotum of a mosquito using a capillary action micro pipette

Figure 4 Wind tunnel configuration, showing the twin fluid gravity fed venturi nozzle, the baffled opening for air straightening, opening downwind from the point of atomization and at the rear an extraction fan.