SOP: Net in tube bioassay – pyrethroid-chlorfenapyr nets

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Related documents

- I2I Best Practice SOP Library, August 2023 (https://innovationtoimpact.org/)

1. Purpose

This SOP outlines the procedures for conducting bioassays to evaluate the efficacy of alphacypermethrin and chlorfenapyr in pyrethroid-chlorfenapyr nets and is modified from an original version written by Seth Irish.

2. Background

Standard cone bioassays with standard susceptible strains will not allow for a separation of the effects of alphacypermethrin and chlorfenapyr. In general, alphacypermethrin will have a rapid action whereas it is thought that chlorfenapyr will take a longer time to affect mosquitoes. Previous studies undertaken by Oxborough et al. (2015) suggest that attaching to treatment nets to filter paper and carrying out a standard World Health Organisation (WHO) tube test, with an exposure time of 30 minutes, may provide results that are more accurate of those being obtained in experimental hut studies using pyrethroid-chlorfenapyr net.

[1] Historical versions of SOPs can be found on the I2I website (https://innovationtoimpact.org/)

Mosquitoes used for this bioassay must be adult females aged 3-5days old that are non-blood-fed. They should be sugar starved for approximately 3 hours prior to assay. 20-25 mosquitoes will be tested per replicate tube, with a minimum of 4 tubes tested per net treatment, per day for the two treatment nets, and 2 replicate tubes for the control untreated net. This will give a minimum of 200-250 mosquitoes tested per day.

All testing must be carried out under dark conditions and during the 'night phase' of a mosquito's circadian rhythm. This can either be done at night-time, between the hours of 6pm and 12am, or using mosquitoes that have been reared in an insectary under altered day/night cycles, thus allowing testing to be completed in the afternoon. Environmental conditions in the testing room should be 27°C ± 2°C with relative humidity (RH) of 75% ± 10%. All testing conditions need to be recorded each day as standard procedure.

Treatment nets will be cut and attached to filter paper and must be stored in the fridge (4°C) prior to- and post-assay. They need to be removed and placed in the testing room for a minimum of 1hour prior to testing to reach ambient temperature.

3. Materials and equipment

- 10 sets of WHO cylinder tubes
- Aspirators – separate aspirators for introduction and removal of mosquitoes from tubes
- Scissors
- Cups
- Rubber bands
- Untreated netting
- Cotton wool
- Timers
- 10% sugar solution
- Stapler
- Permanent markers
- Record sheets

- Gloves
- Filter paper
- Alphacypermethrin only net
- Pyrethroid-chlorfenapyr net
- 200-250 non-fed, 3-5day old female mosquitoes

4. Procedure

1. The testing of chlorfenapyr bioefficacy is challenging and requires either a wild resistant strain or laboratory-reared resistant strain. The use of a resistant strain introduces considerable variability into the bioassay, as even laboratory resistant strains can vary in their degree of resistance. For this reason, before each bioassay, the susceptibility of the strain to be used should be assessed in WHO tube tests using the alphacypermethrin. The results of the initial bioassay can be recorded in a worksheet as shown in Table 1.

Table 1. Results of WHO tube test to assess the alphacypermethrin susceptibility of the resistant strain.

Mortality	Time	Tube 1	Tube 2	Tube 3	Tube 4
Wild strain	24hr				
	48hr				
	72hr				
Control	24hr				
	48hr				
	72hr				

2. If the resistant strain shows a mortality of at most 70% then it can be used for testing of chlorfenapyr in pyrethroid-chlorfenapyr net testing. If this threshold is not met, either another field strain should be used after testing, or the net pieces should be tested at a laboratory that maintains a suitable strain of resistant mosquitoes. Note that it is not necessary that the vector strain be used, as the tests are meant to assess the quantity of insecticide on the netting, not the susceptibility of the mosquitoes. Many field sites may have easier access to *Culex quinquefasciatus* or *Aedes aegypti*

that meet these criteria and use of these strains are encouraged. Once a suitable resistant strain has been found, it can be used in bioassays.

3. Four 12x15cm pieces of pyrethroid-chlorfenapyr net will be cut out from the whole net to be tested and attached to separate 12x15cm pieces of filter paper using a stapler. In the same way, four pieces of netting treated with alphacypermethrin at 100mg/m^2 will be attached to another four separate pieces of filter paper.
4. The tests will be carried out according to WHO procedures (WHO, 2016). Exceptions to the WHO protocol are in bold. **All stages of the assay must be carried out under dark conditions during the 'night phase' of the mosquito circadian rhythm and testing conditions of 27°C ± 2°C and 75% ± 10% relative humidity.**
5. The investigator puts on gloves. Ten sheets of clean white paper (12 × 15 cm), rolled into a cylinder shape, are inserted into ten 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.
6. 200-250 active female mosquitoes are aspirated (in batches) from a mosquito cage into the ten green-dotted holding tubes through the filling hole in the slide, to give ten replicate samples of 20–25 mosquitoes per tube.
7. 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 (Table 2).
8. 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.
9. Eight exposure tubes with red dots are prepared in much the same way as the yellow-dotted tubes. Four red-dotted exposure tubes with the **pyrethroid-chlorfenapyr nets stapled to the inside**. 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. **Four additional tubes will contain the alphacypermethrin-treated netting, and these will also be fastened into position with copper spring-wire clips.**
10. 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.

11. Mosquitoes are kept in the exposure tubes, which are set in a vertical position with the mesh-screen end uppermost, **for a period of 30minutes**.
12. At the end of the **30minute** exposure period the mosquitoes are transferred back to the holding tubes by reversing the procedure outlined in Step 10. The exposure tubes are detached from the slide units (Figure 1).

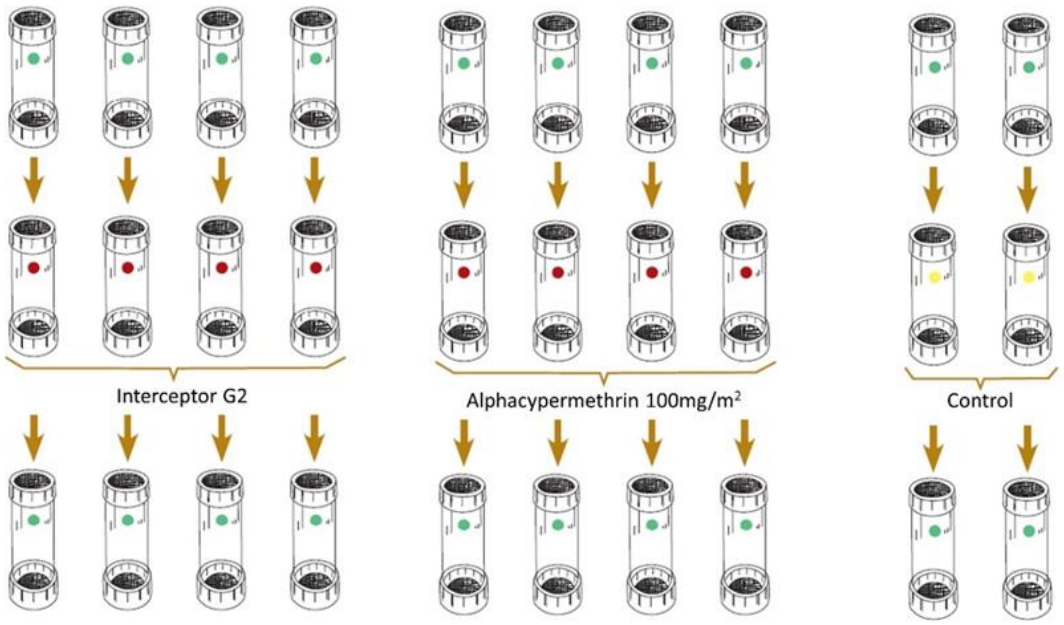

Figure 1. Representation of tests to determine the bioefficacy of pyrethroid-chlorfenapyr nets.

13. Knock down (KD) is scored 60 minutes after the assay has ended and then a pad of a cotton wool soaked in 10% sugar water is placed on the mesh-screen end of the holding tubes.
14. Mosquitoes are maintained in the holding tubes for **72 hours**. **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 and 75% ± 10% relative humidity**. This is very important, and an incubator should be considered if these conditions cannot be maintained. Temperature and humidity must be recorded during the recovery period.
15. **At 24, 48, and 72h**, the number of dead mosquitoes is counted and recorded (Table 3). 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 (Table 2).

5. Data collection

Table 2. Definition of alive, knocked down and dead mosquitoes (Taken from WHO., 2016).

Alive	Knocked down or dead after exposure	
	Moribund	Dead
– Can both stand and fly in a coordinated manner.	– Cannot stand (e.g., only has one or two legs), – Cannot fly in a coordinated manner, – Lies on its back, moving legs and wings but unable to take off, – Can stand and take off but rapidly falls down.	– No sign of life, – Immobile, – Cannot stand.

Table 3. Example of data recording sheet for bioassays in WHO cylinders.

Treatment	Time	Tube 1	Tube 2	Tube 3	Tube 4
Pyrethroid-chlorfenapyr net	Number tested				
	KD 60mins				
	Dead 24hrs				
	Dead 48hrs				
	Dead 72hrs				
Alphacypermethrin net	Number tested				
	KD 60mins				
	Dead 24hrs				
	Dead 48hrs				
	Dead 72hrs				
Untreated control	Number tested				
	KD 60mins				
	Dead 24hrs				
	Dead 48hrs				
	Dead 72hrs				

Knock-down, mortality and adjustment calculations

Knock down (KD) is scored at 60minutes post-exposure and is calculated by summing the number of knocked-down mosquitoes across all exposure replicates and then expressing this as a percentage.

Observed knock-down = (total number of KD mosquitoes / total sample size) x 100

The assessment of mortality is made at 24hours, 48hours and 7hours post-exposure.

The mortality of the test sample is calculated by summing the number of dead mosquitoes across all exposure replicates and then expressing this as a percentage.

Observed mortality = (total number of dead mosquitoes / total sample size) x 100

A similar calculation should be made in order to obtain a value for the control mortality. If the control mortality is ≥20%, the tests must be discarded. When control mortality is ≤20%, then the observed mortality must be corrected using Abbott's formula as follows;

Correct mortality = [(% observed mortality - % control mortality) / (100 - % control mortality)] x 100

If the control mortality is <5% (i.e. one dead mosquito out of 25), no correction of test results is necessary.

Abbott's formula will also be used to estimate the effect of chlorfenapyr, adjusting for the mortality due to alphacypermethrin. This estimate will be made using the following formula and using mortality recorded at 72hours post-assay.

Let X = % mosquitoes alive in the alphacypermethrin tubes

Let Y = % mosquitoes alive in the chlorfenapyr tubes

Corrected mortality due to chlorfenapyr = [(X-Y)/X] x 100

When reporting mortality counts, the sample size should always be given and an estimate of the 95% confidence intervals.

6. Glossary of terms

KD	Knock-down
RH	Relative humidity
SOP	Standard operating procedure
WHO	World Health Organisation

7. References

Oxborough, R. M., N'Guessan, R., Jones, R., Kitau, J., Ngufor, C., Malone, D., Mosha, F. W., & Rowland, M. W. (2015). The activity of the pyrrole insecticide chlorfenapyr in mosquito bioassay: Towards a more rational testing and screening of non-neurotoxic insecticides for malaria vector control. *Malaria Journal*, *14*(1). https://doi.org/10.1186/s12936-015-0639-x

WHO. (2016). *Test procedures for insecticide resistance monitoring in malaria vector mosquitoes Second edition*.