Tunnel Test

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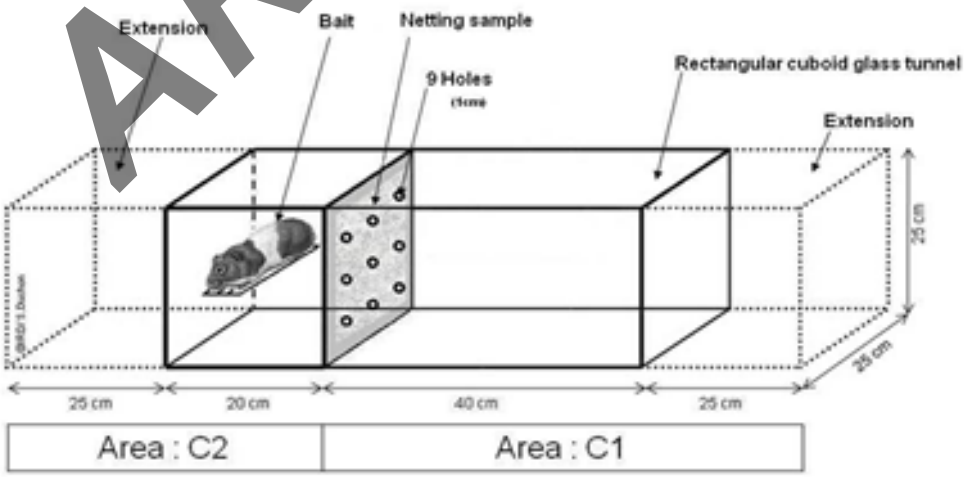
WHOPES Guidelines: Guidelines for laboratory and field testing of long-lasting insecticidal nets

The efficacy of treated nets may be underestimated if judged based on the outcome of standard cone bioassays. This is true particularly for insecticides that have a high excito-repellent effect, such as permethrin and etofenprox. In such cases, the efficacy (mortality and blood-feeding inhibition) of LNs washed 20 times or more that no longer meet the criteria in standard cone bioassays should be studied in a tunnel in the laboratory. The netting piece that results in mortality closest to the mean mortality in the cone bioassay is used in the tunnel test. The tunnel test is used to measure the mortality and blood feeding success of host-seeking mosquitoes in an experimental chamber. The assay is carried out in a laboratory by releasing non-blood-fed female anopheline mosquitoes aged 5–8 days into a 60-cm tunnel (25 cm x 25 cm square section) made of glass (see the diagram below). At each end of the tunnel, a 25-cm square cage covered with polyester netting is fitted (extension). The LN netting sample, held in a disposable cardboard frame, is placed at one third the length of the glass tunnel. The surface of netting available to the mosquitoes is 400 cm2 (20 cm x 20 cm), with nine holes 1 cm in diameter; one hole is located at the centre of the square, and the other eight are equidistant and located 5 cm from the border. In the shorter section of the tunnel, a suitable bait (e.g. guinea-pig or rabbit) is placed, which is unable to move and is available for mosquito biting. One hundred female mosquitoes are introduced into the cage at the end of the longer section of the tunnel. They are free to fly in the tunnel but have to make contact with the piece of netting and locate the holes in it before passing through to reach the bait. After taking a blood meal, the mosquitoes may fly back to the cage at the end of this compartment and rest. A tunnel with untreated netting is always used as a negative control.



During the tests, the tunnels and cages are held at 27 ± 2 °C and 75% ± 10% relative humidity at night in full darkness. After an exposure of 12–15 h, the mosquitoes are removed from each section of the tunnel with a glass suction tube and counted separately; mortality and blood-feeding rates are recorded. Blood-feeding inhibition is assessed by comparing the proportion of blood-fed females (alive or dead) in treated and control tunnels. Overall mortality is measured by pooling the mortality rates of mosquitoes from the two sections of the tunnel. Mortality on the LNs should be corrected for mortality in the controls with Abbott's formula. If mortality in the controls is > 10%, the test should be considered invalid. As blood-feeding by controls has a considerable effect on mortality in the presence of treated samples (i.e. the host-seeking behaviour increases the chance of contact with treated fabric), a 50% minimum cut-off value of the blood-feeding rate in controls should be established for tunnel tests. A sample data collection sheet for tunnel tests is provided in Appendix 1.

Procedure:

1. **Test conditions**
    a. Non-blood-fed female anopheline mosquitoes aged 5–8 days should be used for all tunnel tests.
    b. Put the cage(s) containing the mosquitoes in the testing room for 1 hour.
    c. Record temperature and humidity in the acclimation room before and after acclimation.
    d. Remove glucose-soaked cotton balls for acclimatization 1 hour before the test.

2. **Preparation of tunnel and materials**
    a. Keep tunnels used for control tests and tunnels used for the testing of net samples containing insecticides separate. Label the tunnels to identify those used for controls.
    b. Wipe clean the glass section of the tunnel with a 70% ethanol solution at least 3 hours before preparing tests. Wipe clean with a paper towel dampened with water to finish decontamination.
    c. Soak aspirators in 10% bleach solution and rinse twice with tap water. Leave to dry on a paper towel.
    d. Switch on the humidifier and heater as necessary to achieve correct temperature and humidity for the test 1 hour before testing. Set temperature to 27 ± 2°C and 75 ± 10% RH unless otherwise stated.

3. **Preparation of the net samples for testing**
    a. Put on gloves and lab coat.
    b. Obtain the 25cm x 25cm treated net samples from the storage area.
    c. Record the net code on the form. Double check that the net code matches the code required in the protocol.
    d. Set and staple the net piece on the cardboard frame. If using a metal frame, attach the net piece in the metal frame.

e. Make 9 holes on the sample. Centre hole is placed in the middle of the sample area and 8 other holes are equidistant and located at 5cm from the border. Use a template if necessary
f. Insert the frame into the slot at the centre of the glass tunnel.
g. Repeat steps a-f with a control net piece in a control tunnel. Change gloves between treated net piece and control.
h. Attach the cage extensions to the end of each the tunnels. Use either rubber bands or masking tape to fasten the ends, ensuring the netting is as close to the tunnel as possible leaving no gaps.
i. Label each tunnel with masking tape with the protocol number, net piece ID, replicate number, and technician initials.

4. Preparation of the animal bait

a. Shave a guinea pig and immobilize it in a mesh cage.
b. Introduce the guinea pig in the appropriate compartment of the tunnel.
c. Put paper towel under the guinea pig cage to catch urine and faeces or place the mesh cage into a plastic container.

5. Preparation of the test system

a. Aspirate mosquitoes into paper cups or disposable cups for each tunnel
b. Maintain the mosquitoes for 1 h after aspiration on bench for acclimatisation.
c. Remove and replace any unfit or weak mosquitoes.
d. Label cups with protocol code, date, and technician initials.
e. Record temperature and humidity in the acclimation room before and after acclimation.

6. Release of mosquitoes in tunnel

a. Put all the mosquitoes in the four cups (100 mosquitoes total) into the compartment 1 of the tunnel (see the diagram above).
b. Open and release mosquitoes cup after cup into the appropriate compartment at 6:00PM. Record the actual time that mosquitoes are introduced into each tunnel.
c. Ensure temperature and humidity are set and maintained at 27 ± 2°C and 75 ± 10% RH (unless otherwise stated in the study plan). Record temperature and humidity, data logger ID, and technician initials.
d. At 9:00AM the following day (15 hours of exposure in the tunnels), remove mosquitoes from each section of the tunnel and count separately. Record the time when the last mosquitoes are removed from the tunnel. Record the temperature and humidity, data logger ID, and technician initials.
e. Put on gloves and starting with the bait chamber, remove all dead mosquitoes with a pair of forceps. Use an aspirator and remove live mosquitoes from the bait chamber.

Collect the mosquitoes in several holding cups (not more than 15 per cup), for blood fed and for unfed. Put a glucose-soaked cotton ball on the top of the cup netting. Label the cups with protocol code, date of exposure, net piece ID, section of tunnel, and abdominal status.

f. Repeat mosquito collection process for the release chamber as described above.
g. Record the number of mosquitoes tested, alive and blood-fed, dead and blood-fed, alive and unfed, dead and unfed in the bait chamber and release chamber using the proforma data record form (Appendix 1).
h. Repeat steps c-e for each tunnel. Record the time when the last mosquitoes are removed from each tunnel. Change gloves, aspirators and forceps between each tunnel if they are different treatments.
i. Put on a new pair of gloves and remove the metal or cardboard frame containing the net piece from the tunnel. Wrap the net piece in aluminium foil and return it back to the storage room. Repeat the process for all net pieces ensuring gloves are changed each time.
j. Wash tunnel first with a 5% solution of Decon, rinse thoroughly with water and then wipe with paper towels soaked with 70% ethanol.
k. Decontaminate work surfaces.
l. Discard results if control mortality exceeds 10% and/or if blood-feeding in the control is <50%.

7. Post-exposure period

a. Ensure that the holding room of incubator have been set to 27 ± 2 °C and 75% ± 10% relative humidity.
b. Put cups on test room bench or in incubator depending on availability.
c. Record temperature and humidity post-exposure using a calibrated data logger.
d. Observe outcomes at the intervals specified in the study plan and record the data on the appropriate form.

Appendix 1. Raw data recording form

Tunnel bioassay of nets collected in households

Name of person performing bioassays: ……...

Date of test (dd/mm/yyyy): |__|__|/|__|__|/|__|__|__|__|

LN code: |__|__|__|__|__|__|__|__|

Temperature: |__|__| °C Relative humidity: |__|__| %

Test mosquito species and strain: …….

Age of mosquitoes: |___| days

Test start time* (h/min): End time (h/min):

* Females are introduced at 18:00 h and collected at 09:00 h.

		Blood-fed females		Unfed females		Total	
		Alive	Dead	Alive	Dead	Alive	Dead
Control	Compartment 1						
	Compartment 2						
	Total						
Treatment (LN)[a]	Compartment 1						
	Compartment 2						
	Total						

Compartment 1, long section of tunnel into which mosquitoes are released (see Figure above; area C1); compartment 2, section between test netting and animal bait

[a] Add additional treatment rows when more than one subsample of the same net or samples of other nets are tested in parallel.