SOP: Tunnel test

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1	30/10/2020	Angus Spiers	I2I
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Version Control[1]

Version	Date	Updated by	Description of update(s)
2	April – May 2022	Alex Wright, Natalie Lissenden	Purpose, materials & equipment, post-collection holding for nets containing PPF, data collection sheet information, glossary of terms and references added.
3	August 2024	Annabel Murphy-Fegan	Background, materials & methods, procedure updated. Data collection sheet added.

Related documents

- I2I Best Practice SOP Library, 30 October 2020 (https://innovationtoimpact.org/)
- WHO Prequalification of Vector Control Products .Bioassay methods for insecticide-treated nets: tunnel test. (WHO, 2023)
- WHO Guidelines for laboratory and field testing of long-lasting insecticidal nets (WHO, 2013)

1. Purpose

The tunnel test is used to investigate the biological activity of a material's surface (untreated or treated with an active ingredient) under controlled laboratory conditions. The exposure of mosquitoes at a net interface in the wind tunnel is representative of interaction with the test material and observations are made on the relevant effects (e.g. mortality and blood feeding success) on mosquitoes within a host seeking experimental chamber. Nets washed at least 20

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

times that do not meet the criteria in the WHO cone test in laboratory should undergo tunnel tests. This SOP details how to conduct a tunnel test for laboratory testing.

2. Background

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 LLINs 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 assay is carried out in a laboratory by releasing non-blood-fed, female anopheline mosquitoes aged 5–8 days into the tunnel made of glass/acrylic (see Figure 1). The tunnel comprises three chambers 1) netted release chamber (25 x 25 x 25cm), 2) response chamber (60 x 25 x 25cm with a volume of 37,500cm^3) and 3) netted collection chamber (25 x 25 x 25cm). The treated net sample to be tested is attached to a 25 x 25cm frame (e.g. cardboard) and slotted into the WHO tunnel. 50 mosquitoes are introduced into the cage and are free to fly. The mosquitoes must pass through a holed netting sample to reach an animal bait (e.g. guinea pig or rabbit) for mosquito biting. 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.

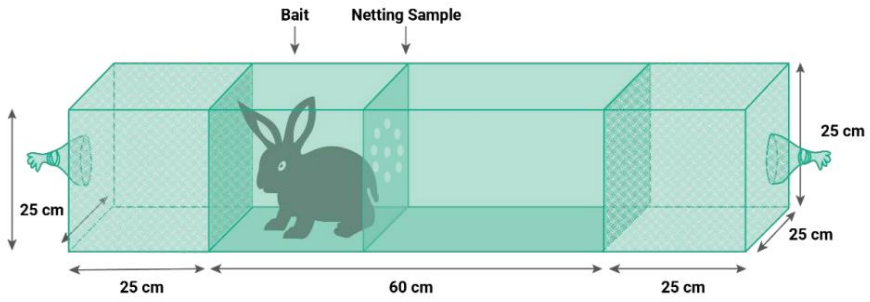

Figure 1: Tunnel test

During the tests, the tunnels and cages are held at 27°C ± 2°C and 80% ± 20% relative humidity (RH) at night in full darkness. After an exposure of 12–15 hours, 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 LLINs should be corrected for mortality in the controls with Abbott's formula. If mortality in the controls is >10% at 24 hours or >20% for extended holding times 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.

3. Materials & Equipment

- Prepration of laboratory room
    - Calibrated data logger
    - Environmental condition record sheet
    - 70% ethanol solution
    - Labels
    - Marker pen
    - Paper towel
    - 10% bleach solution
    - Humidifier
    - Heater (if necessary)
- Preparation of sample
    - Gloves
    - Lab coat
    - Sample recording form
    - Metal or Cardboard frame

    - Stapler
    - Scissors
    - Rubber bands
    - Masking tape
- Preparation of animal bait
    - Shaving machine
    - Mesh cage for bait animal
    - Paper towel
    - Plastic container
- Preparation of test system
    - Paper cups
    - Aspirator
    - Marker pens
    - Control netting pieces
    - Rubber bands
    - Data logger
    - Record sheet
- Release of test system (in addition to above materials)
    - Two paper cups (25 mosquitoes/cup)
    - Aspirator
    - Gloves
    - Forceps
    - Paper cups (with netting and rubber bands)- not more than 15 mosquitoes/cup
    - Record form
    - Aluminum foil
    - Decon solution
    - 70% ethanol
- If using PPF-net
    - One cup per live mosquito

- Netted plastic cups
- Larval food
- Recording sheet

4. Procedure

1. **Test conditions**
    a. Switch on the humidifier and heater as necessary to achieve correct temperature and humidity for the test one hour before testing:27°C ± 2°C and 80% ± 20% RH unless otherwise stated.
    b. Non-blood-fed female anopheline mosquitoes aged 5–8 days should be used for all tunnel tests. Remove access to sugar solution 6-12 hours prior to testing. The exact time is strain dependent and determined by the time required to ensure there is 50% blood feeding success in the controls.
    c. Put the cage(s) containing the mosquitoes in the testing room for one hour.
    d. Record temperature and humidity in the acclimation room before and after acclimation.
    e. Remove glucose-soaked cotton balls for acclimatization 6-12 hours 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/acrylic section of the tunnel with a 70% ethanol solution at least three 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.

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. On each net sample piece, use a hole punch or scissors to cut nine holes 1cm in diameter (ensure all holes are standardised in size), one located in the centre and 8 located 5cm from the border (see Figure 2). A template can be used if necessary.
e. Set and staple the net piece on the cardboard frame. If using a metal frame, attach the net piece in the metal frame.
f. Insert the frame into the slot at the center of the glass/acrylic 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.

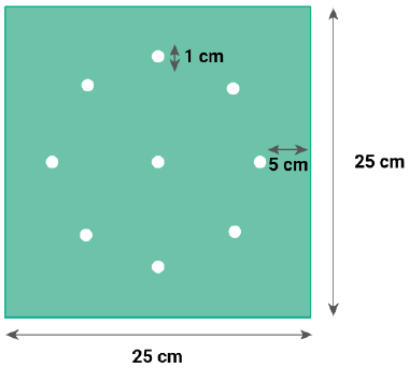

Figure 2: Test material with 9 holes required for tunnel test

4. Preparation of the animal bait

a. Shave a patch on the back of the bait animal and immobilize it in a mesh cage.
b. Introduce the animal in the appropriate compartment of the tunnel.
c. Put paper towels under the mesh cage to catch urine and faeces or sit the mesh cage in a plastic container.

5. Preparation of the test system

a. Aspirate mosquitoes into paper cups or disposable cups, one for each tunnel. Do not select mosquitoes that are ae small, inactive or missing legs/wings.

b. Maintain the mosquitoes for 1 hour after aspiration on bench for acclimatisation.
c. Label cups with protocol code, date, and technician initials.
d. 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 cups (50 mosquitoes total into the compartment one of the tunnel (see Figure 1).
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°C ± 2°C and 80% ± 20% 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% at 24 hours and 20% at an extended time period 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°C ± 2°C and 80% ± 20% RH
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.

8. Post-collection holding for nets containing PPF (Ngufor et al., 2014)

a. After scoring mortality (24 hours post-collection from the experimental huts), separate the live blood-fed mosquitoes of each treatment in separate cages and provide access to a second blood meal.
b. Once gravid (within 2-3 days), chamber individual mosquitoes separately in their own netted plastic cups containing approximately 50mL of fresh water.
c. Monitor daily for eggs and record the number of eggs laid by each female mosquito for up to nine days. Add a pinch of larval food to any chamber which contains eggs.
d. Record the number of larvae (L2) which hatched for another 4-6 days.

9. Collection and reporting of data

Refer to the data collection sheet in Appendix 1 for recording data.

5. Glossary of terms

LLIN	Long lasting insecticidal net

PPF	Pyriproxyfen
RH	Relative Humidity
SOP	Standard Operating Procedure
WHO	World Health Organization

6. References

Ngufor, C., N'Guessan, R., Fagbohoun, J., Odjo, A., Malone, D., Akogbeto, M., & Rowland, M. (2014). Olyset Duo® (a pyriproxyfen and permethrin mixture net): An experimental hut trial against pyrethroid resistant Anopheles gambiae and Culex quinquefasciatus in southern Benin. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0093603

WHO. (2013). Guidelines for laboratory and field-testing of long-lasting insecticidal nets. In *WHO/HTM/NTD/WHOPES/20131*. World Health Organization.

7. Appendix 1: Example record data sheet

Protocol code		Mosquito species and strain	
Date of exposure		Age of mosquito	
Test item ID number		Abdomial status	
		Exposure conditions	Time / Day/Night

Data logger ID number	Exposure start		Exposure end		24 hours		48 hours		72 hours	
	Temp °C	Humidity (%)	Temp °C	Humidity (%)	Temp °C	Humidity (%)	Temp °C	Humidity (%)	Temp °C	Humidity (%)
Staff initials										
Time										

Replicate	Compartment	Insecticide (and ID number)	Exposure start time	Exposure end time	KD/Dead end test	KD/Dead 24 hours	KD/Dead 48 hours	KD/Dead 72 hours	Unfed/bloodfed 24 hours	Unfed/blood fed 48 hours	Unfed/bloodfed 72 hours	Total mosquitoes at Test End
1												
2												
3												
4												
5												
6												
7												
8												
					Start time							
					Date							

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

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