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Standard operating procedure (SOP) for the exposure of *Culicoides* biting midges to an infectious blood meal under biosafety laboratory 3 conditions (BSL3)

For a video on the basic technique of blood-feeding of *Culicoides* through a membrane see video …. on YouTube (Alec Hochstrasser).

Material:

- *Culicoides.* Field-collected (Paslaru et al., 2018) or laboratory-reared *C. nubeculosus* or *C. sonorensis* (Boorman, 1974)
- Anticoagulated (heparin, EDTA) blood (cow, rabbit, other) (defibrinated blood might work as well)
- Anesthesia chamber (Fig. 1)
- Feeding chamber (Fig. 2)
- Safety transport container with screw lid (e.g. Bio-bottle, New Zealand) (Fig. 3)
- Parafilm (or Nescofilm)
- Mouth aspirator with HEPA Filter (Model 612; John W. Hock Company, USA)
- Waste plastic bag
- 400 ml glass beaker (or large petri dish; serving as water bath)
- Blood bath cup (Fig. 4) (Proben-Becher PP; 180 ml; article Nr. 4438, Semadeni AG, Switzerland)
- Heating-stirring plate with stirring magnet
- Glovebox
- Mechanical insect aspirator (InsectaVac aspirator, BioQuip Products, USA)
- *Culicoides* maintenance box (Fig. 5)
- Insect rearing cage (BugDorm-4M3030, W32.5 x D32.5 x H32.5 cm, BugDorm Store, Taiwan).
- Falcon tube (50 ml)
- CO_2 pressure tank with regulator valve
- CO_2 pad (FlyPad, Flystuff, USA) in glove box
- Axial fan (e.g. X-Fan RAH8025S1)
- Disinfectant (Virkon S; Provet AG, Switzerland) and 70% ethanol
- Magnifying lamp (Lupenleuchte, Conrad Electronic AG, Wollerau, Switzerland) or binocular/monitor (RyEcoCam HD Digitalmikroskop; Ryf, Grenchen, Switzerland)
- Precision tweezers
- Cotton wool
- 5% glucose solution
- Autoclave bag
- Ice packs (at -20 °C)
- Laminar flow CLII
- Climate chamber

Figure 1: Anesthesia chamber*

Figure 2: Feeding chamber*

Figure 3: Safety transport container

Figure 4: Blood bath cup, shown with feeding chamber inside

Figure 5: *Culicoides* maintenance box*

*detailed description see below

Anesthesia chamber:

Cut a hole with a diameter of appr. 13 mm (for adding *Culicoides* with a mouth aspirator) into the side of a cardboard drinking cup. Prepare a stopper made from cotton to close the hole (for better stability wrap the cotton with tape). Cover the cup with a piece of net (e.g. curtain fabric; choose a small mesh size so *Culicoides* cannot escape) and fix it with a rubber band. Chamber can be re-used.

Feeding chamber:

Cut out the bottom of a 30 ml Nalgene™ Wide-Mouth Straight-Sided PPCO Jar (ThermoFisher Scientific AG, Switzerland). This end will be sealed with Parafilm or Nescofilm (feeding membrane) during the bloodmeal. Cut a hole in the lid (diameter appr. 20 mm) and cover it at the inside with a net (e.g. curtain fabric; choose a small mesh size so *Culicoides* cannot escape). Fix the net with hot melt adhesive (hot glue). The feeding chamber can be re-used.

***Culicoides* maintenance box:**

Cut a hole with a diameter of appr. 7 mm (for adding *Culicoides* with a pair of tweezers) into the side of a 64 mm Card box (Watkins & Doncaster, UK). The lid of the box consists of a cardboard ring and a cardboard disk; discard the cardboard disk. Cover the box with a net (e.g. curtain fabric), choose a small mesh size that *Culicoides* are not able to escape) and fix it with the cardboard ring. Finally, fix the whole net and lid with some layers of tape (not depicted in Fig. 5). Prepare a stopper made from cotton to close the hole (for better stability wrap the cotton with tape). For a secure closure of the hole, fasten the stopper with tape as soon as the *Culicoides* are inside. Discard the box after use.

Preparation

These steps can be done under BSL1 or 2 conditions.

Blood meal

- Add 35 ml blood to 50 ml Falcon tube, fill up with 1 x PBS, centrifuge at 2000 x g 15 min at RT
- Discard supernatant with disposable plastic pipettes and replace with equal volume of PBS.
- Wash the pellet (blood cells) 3 times with PBS as above
- Discard supernatant and store sediment at 4 °C for a maximum of 24 h before use

Culicoides

- Field-collected *Culicoides* are kept for at least three days in a climate chamber with access to 5% glucose solution. Before exposure to the infectious blood meal *Culicoides* are starved for 24 hours.
- Seal the bottom of a feeding chamber with a highly stretched layer of Parafilm or Nescofilm (feeding membrane).
- With a mouth aspirator, transfer the *Culicoides* into the anesthesia chamber through the side opening (non-Culicoides can be discriminated by eye).
- Anesthetize them by placing the cup at -20 °C for at least 120 seconds (alpine species may require up to 4 minutes)
- Remove the net from the anesthesia chamber and insert the *Culicoides* into the feeding chamber through its screwable lid (maximum 300 specimens)
- Transfer the feeding chamber containing the *Culicoides* into a safety transport container. Place this container together with the Falcon tube containing the washed blood in a polystyrene box for transportation to the BSL3 facility.

Inoculation (BSL3)

In the glovebox, make sure that the CO_2 Flypad is working (connection to CO_2 pressure tank). Transfer some precooled ice packs into the glovebox (might be needed to keep the insects cool after feeding and before using CO_2). Use CO_2 very carefully (intermittently, at low dose) to avoid high concentrations inside the box that could kill *Culicoides*. Make sure that during the sorting of live *Culicoides* there are periods when the CO_2 is off when the *Culicoides* are well sedated.

Do not leave CO2 supply on constantly as this might kill the Culicoides: the CO2 should be used.

Oral feeding of Culicoides

- Preparation of the infectious blood meal (to be performed in a laminar flow CLII cabinet):
    - Allow the blood cells of the washed blood to sediment in the Falcon tube for approx. 20 min, and then discard the top layer (transparent yellowish fluid, serum) using disposable plastic pipettes.
    - Prepare the inoculum (blood spiked with pathogen, total volume minimum 10 ml): inside the CLII cabinet mix the appropriate amount of blood sediment with the pathogen-containing solution at he required ratio (e.g. 1:2, 1:10, depending on the pathogen concentration required. The lower the blood dilution the better is the feeding rate).

    - Transfer the infected blood into the blood bath cup (Fig. 4), containing a magnet to mix the blood during feeding. Make sure the stirring magnet is totally covered by the blood.
- Feeding Culicoides:
    - With a strip of tape, create a handle on the lid of the feeding chamber (but do not cover the hole in the lid completely, air must be able to circulate through the net!) Place the feeding chamber containing the midges into the blood bath chamber (make sure that the feeding chamber is not touching the stirring magnet otherwise the membrane could rip. When using 10 ml of blood as specified above, the feeding chamber should be floating well above the magnet). Close the blood bath cup with its lid.
    - Partially submerge the blood bath cup containing the feeding chamber inside the 400 ml beaker containing water previously warmed to +25-30 °C (use a thermometer to check the temperature). To prevent it from floating, pinch the blood bath cup in the beaker with the help of sponge or foam material. Place all these three containers on a heating magnetic stirrer pre-warmed to +25-30 °C.
    - Switch on the magnetic stirrer at medium power. Make sure that the magnet is moving freely.
    - Leave the *Culicoides* to feed for 45 minutes.
- After feeding
    - When the time has elapsed, transfer the blood bath cup with the feeding chamber to the glovebox.
    - Remove the feeding chamber from the blood bath, wipe off the blood at the outside with a paper towel and place the feeding chamber inside a plastic waste bag. Close the bag with tape and wipe its outer surface with disinfectant and then with 70% ethanol.
    - Transfer the plastic bag inside the safety transport container to the -20 °C freezer and anesthetize the *Culicoides* for at least 120 seconds (visually check if the *Culicoides* are still active; alpine species may need up to 4 minutes). Afterwards transfer the bag back in the transfer box to the glovebox.
- Sorting the blood-fed *Culicoides* (to be done inside the glovebox)
    - Remove the feeding chamber from the plastic bag and pour the *Culicoides* onto the FlyPad CO_2 anesthesia table (see above for a note of caution).
    - A second person should assist and check for insects flying inside the glovebox which are to be killed.
    - Through the magnifying lamp or the binocular/monitor, collect all the fully engorged *Culicoides* (do not harvest partially engorged ones) by using tweezers and transfer them into a maintenance box (Fig. 5) through the side opening.
    - Place a cotton pad soaked in 5% glucose solution (to be replaced every second day) on the net of the maintenance box which is placed inside a bugdorm. Incubate the midges in a climatic chamber under appropriate temperature, light and humidity conditions.
- Non-engorged *Culicoides* killed by exposing to -80 °C for at least 1 day or they can be discarded in a bottle containing 70% ethanol.
- Switch off the CO_2 supply and remove the CO_2 that has accumulated in the glovebox with a fan

Decontamination:

- The infectious blood can be discarded in an appropriate “liquid waste” bottle.
- The blood bath cup has to be decontaminated as follows: add disinfectant (Virkon) to a volume higher than the blood volume was. Close the lid, wipe the outside surface with disinfectant, transfer it from the glovebox to a Class II cabinet and leave for at least 30 minutes. Make sure that this container is appropriately labelled with a sticker indicating pathogen type, date, time and worker name.

- After the decontamination time, pour the liquid material into a glass bottle (containing Virkon) inside the cabinet, close it with a lid and label it with autoclave tape as “liquid waste”. Rinse the empty container with more disinfectant (Virkon, then ethanol) and discard it in the same “liquid waste” bottle.
- After washing and rinsing, wipe it again with 70% ethanol then double bag it in a plastic bag for autoclaving.
- The interior of the glovebox must be cleaned and disinfected with Virkon. Avoid the use of ethanol as it may damage the Plexiglas walls. Any material taken out of the glovebox also must be wiped with disinfectant and incubated for at least 30 minutes in the CL II cabinet.

Troubleshooting

If any problems are experienced while following this protocol the immediate supervisor should be consulted in the first instance. If unavailable, advice from the Head of Group or any other suitably competent or experienced personnel should be sought.

Waste disposal and inactivation

Transports of material to the autoclave must be done in double containers with absorbable material.

- Reusable and disposable material

All reusable material (e.g. tweezers) need to be properly disinfected, wiped with 70% ethanol and prepared for autoclavation.

Any sorts of tools and containers that cannot be reused must be autoclaved in double bags and then disposed.

- Liquids

Liquid material containing pathogens is transferred into a glass bottle and mixed with disinfectant (Virkon™) for at least 30 minutes and then autoclaved.

Applicable documents

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Paslaru, A.I., A. Mathis, P.R. Torgerson, E. Veronesi (2018): Vector competence of pre-alpine Culicoides (Diptera: Ceratopogonidae) for bluetongue virus serotypes 1, 4 and 8. Parasit Vector 11: 466. https://doi.org/10.1186/s13071-018-3050-y.

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