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Culturing bacterial members of the microbiota from mosquito midguts

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We use this protocol and it's working

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Abstract

This protocol outlines the steps for dissecting adult mosquito midguts, culturing associated bacteria, determining Colony Forming Units (CFUs), and isolating different bacterial types for characterization and sequencing.

Materials

Equipment

- Ice bucket
- Homogenizer
- Pestles
- Vortex
- Colony counter (optional, for counting)
- Forceps
- 200 µl pipettor and tips
- 10 µl pipettor and tips
- 1000 µl pipettor and tips
- Centrifuge

Consumables

- Adult mosquitoes
- Solid media agar plates (at least 3 plates per mosquito, plus extra for contamination checks)
- Test tubes
- Cell culture dish
- Glass petri dish
- Kimwipes
- Spreading beads
- Marker
- Tubes (for dissection/homogenization, filled with 150 µl 1X PBS)
- Tubes (for 1:100 dilution, containing 990 µl 1X PBS)
- Glass slides
- Glycerol (30% for freezer stocks)

Reagents / Solutions

- 70% EtOH
- 1X PBS (sterile)
- Liquid culture media
- Agar (for making solid media)

Dissection

1 Surface Sterilization: Surface sterilize adult mosquitoes from each treatment in 70% EtOH for 30s, then rinse twice in sterile 1X PBS. This can be done in a 6-well cell culture plate using forceps to submerge the mosquitoes.

2 Storage: Leave the adults in 1X PBS until you are ready for dissection.

3 Verification of sterilization: Before dissection, streak one adult mosquito across a solid media agar plate (the same as the media you choose to use in step 11) and incubate at the same conditions as the experimental plates to verify external sterilization. If external sterilization was successful, these plates should grow zero colony forming units (CFUs).

4 Preparation: Clean a microscope slide and forceps with 70% EtOH.

5 Dissection: In a sterile environment (e.g. under a bunsen burner), pipet a small puddle of sterile 1X PBS onto the slide and dissect out the midgut of a single mosquito as shown in: Coleman et al., 2007. Dissection of midgut and salivary glands from Ae. aegypti mosquitoes. J Vis Exp. (5):228. doi: 10.3791/228.

6 Collection: Place each individual midgut in a tube containing 150 µl 1X PBS.

7 Keep Cold: Immediately place the samples on ice and keep them on ice until it is time for homogenization and plating.

Homogenization and Plating

8 Homogenization: Homogenize the dissected guts using a sterile pestle and mechanical homogenizer.

9 Contamination Control: Also homogenize 3-5 samples of 1X PBS that were handled identically to experimental samples but that do not contain midgut tissue to serve as contamination controls.

10 Dilution: Use 10 µl of the midgut homogenate to dilute the sample 1:100 and 1:10,000 in additional sterile 1X PBS.

11 Plating: Plate 50 µl of each homogenate (undiluted and both dilutions) on solid media. Common choices include tryptic soy agar (TSA), Reasoner's 2A agar, lysogeny broth

(LB) agar, blood agar, or gly media. The type of media you choose will dramatically influence the number and type of bacteria that successfully grow. The media you choose will depend on the goals of your experiment and must be carefully considered. For general screening, it is often a wise choice to use more than one media type and to choose media that differ substantially in nutrient composition or amount to ensure capturing as many different isolates as possible. Bear in mind you will require 3 plates per media type per mosquito.

12 Plate incubation: Incubate plates for 3-5 days at room temperature. Different times/temperatures can be used based on the goals of the experiment, but for general screening, we have found that incubation at room temperature for multiple days yields more colony types than incubation at higher temperatures.

CFU Counting and Colony Characterization

13 CFU Count: Count the overall CFU from each midgut.
Note: Make a note of whether there are mixed colonies on the plate.
Dilution Consistency: If more than one dilution gives countable colonies (> 10 but not so crowded they are touching), count all countable plates and average the CFU/ml values obtained.

14 Characterization: Characterize each different type of bacteria growing on the plates using these instructions as a guide:
https://bio.libretexts.org/Learning_Objects/Laboratory_Experiments/Microbiology_Labs/Microbiology_Labs_I/08%3A_Bacterial_Colony_Morphology
Documentation: Take a picture and make a note of all aspects of the morphology of the colony type.

15 Specific Count: Count by hand (or with a colony counter) the number of each different type of colony you characterized in step 13.
Note: If colonies look similar but might represent different genera or species, count them as separate. When isolates are sequenced and definitive identification is obtained (see below), the final determination of colony counts per type can be made.

Isolate Culture and Storage

16 Isolation: Pick representative colonies of each type and, using a sterile loop under a bunsen burner, streak them onto new plates using the same media type(s) as used for the original culturing step. Re-isolate at least once more (ideally twice more) by picking a single colony and re-streaking on a fresh plate to ensure a pure isolate is obtained.

17 Liquid Culture: Grow liquid cultures of each isolate by touching a sterile toothpick to a single colony on the solid media plate and dropping the sterile toothpick into 2ml of liquid media (the same type as used for the solid media) in a sterile test tube. Grow at room temperature with shaking (~200rpm).

18 Freezer Stock: Make 25% glycerol freezer stocks of each isolate for long-term preservation.

18.1 Transfer 1mL of each liquid culture to a microcentrifuge tube. Centrifuge at 3,000xg for 3 minutes to pellet the bacterial cells. Pour off media supernatant and add 1mL sterile 1X PBS. Re-suspend by vortexing. Repeat the centrifugation/wash step two more times. Combine 500µL of washed culture (re-suspended in 1X PBS) with 500µL sterile 50% glycerol solution. Vortex thoroughly to mix and preserve at -80ºC.

Molecular Identification

19 Culture isolates: Re-grow isolates from freezer stock if the previous plates are more than 1 week old.

20 PCR and sequencing: Perform PCR on the 16S rDNA gene from each isolate and sequence the product using Sanger sequencing. A generalized, detailed protocol of this procedure can be found here: https://www.jove.com/v/10510/16s-rrna-sequencing-identifying-bacterial-species-by-pcr
Note: Most bacteria found inside mosquitoes are Gram-negative and therefore are easily lysed by heating. Because of this, we have had good luck using bacterial colonies directly as PCR template, i.e. instead of isolating DNA, we simply set up the PCR and then touch a sterile pipet tip to a single colony, place the pipet tip in the PCR tube, and pipet up and down in the PCR solution to disperse the cells. The initial 95°C denaturation step is enough to lyse the cells (extending it to 3 minutes for just the first cycle can also help with this). This does not work for all colony types, however, and in these cases you would need to extract DNA as described in the linked protocol.

21 Finalizing dataset: Once you have obtained genus/species identification for each colony type, you can return to your colony counts and determine whether any that you initially characterized as different colony types are indeed the same species. These counts should be combined to improve the accuracy of your characterization of the microbial community structure in your mosquitoes.

Protocol references

Coleman et al., 2007. Dissection of midgut and salivary glands from Ae. aegypti mosquitoes. J Vis Exp. (5):228. doi: 10.3791/228.

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